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HEMOLINE: *In vitro* and *In silico* analysis of Kangkong (*Ipomoea aquatica*) extract as blood thinning component for anticoagulation in response to cardiovascular diseases through factor Xa hydrolase (PDB ID: 2P16) inhibition

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Abstract

Cardiovascular diseases (CVDs) are a leading cause of death worldwide, often linked to excessive blood clot formation. While current anticoagulants are effective, they pose bleeding risks and require close monitoring. This study examines the anticoagulant potential of Kangkong *(I. aquatica)* extract by assessing clotting time using PT and aPTT in vitro assays. Results showed that the crude extract significantly prolonged clotting times (42.02s ± 2.72s), surpassing the normal range and exceeding the machine's limit (210s ± 0s), similar to heparin. This suggests *I. aquatica* may delay clotting by partially inhibiting tissue clotting factors in the extrinsic pathway. Additionally, the study explores the extract's ability to inhibit Factor Xa Hydrolase (PDB ID: 2P16) through molecular docking analysis via AutoDock Vina. A total of 70 phytochemicals from *I. aquatica* underwent docking analysis, revealing that Tamarixetin had the strongest binding affinity (-8.7 kcal/mol), followed by Kaempferol (-8.6 kcal/mol) and Isorhamnetin (-8.5 kcal/mol). The top 10 compounds interacted with at least one active site residue of human coagulation Factor X complexed with an apixaban inhibitor, demonstrating strong potential to prevent thrombus formation and inhibit thrombin generation. The research highlights *I. aquatica* as a promising natural anticoagulant, capable of significantly prolonging clotting times and interacting with coagulation-related proteins. These findings support its potential as a plant-based alternative to traditional anticoagulants, paving the way for safer, more accessible therapies for preventing and managing cardiovascular diseases.

Keywords: Cardiovascular Disease; Anticoagulation; Prothrombin Time; Activated Partial Thromboplastin Time; Molecular Docking; Factor Xa Hydrolase; *I. aquatica*; 2P16

1. Introduction

Cardiovascular Diseases (CVDs) are the leading cause of death globally. According to WHO (2021), an estimated 17.9 million people died from CVDs in 2019, representing 32% of all global deaths. Of these deaths, 85% were due to heart attack and stroke. There is a pressing need for countries to develop public health strategies focused on preventing cardiovascular diseases. This includes emphasizing the importance of global efforts to share information and implement health programs, particularly in underserved regions. (American College of Cardiology, 2023) In 2021, the Philippines reported coronary heart disease (CHD), such as stroke, and cancers as the three leading causes of death. CHD alone accounted for over half a million cases, representing approximately 19% of all deaths in the country which fall under CVDs, This marks an increase from the first half of 2020, contributing to around 17% of total fatalities (Philippine Statistics Authority, 2021).

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Blood coagulation involves a complex sequence of reactions that require many proteins (factors) to function in a specific order for successful clot formation. This process is quick and efficient, but it necessitates careful regulation; without it, excessive clotting may lead to thrombosis (Belur et al., 2024). Thrombosis is a significant complication associated with cardiovascular disease, resulting in conditions such as myocardial infarction, acute ischemic stroke, and venous thromboembolism. It occurs when a thrombus forms within blood vessels, obstructing blood flow (Alkarithi et al., 2021). Studies by Alkarithi et al., 2021, Wendelboe & Raskob, 2016 supports that the majority of heart attacks and strokes result from thrombus formation that obstructs blood flow to critical organs.

Factor Xa is a crucial enzyme in the coagulation cascade, primarily responsible for converting prothrombin into thrombin, a key step in the formation of blood clots. Due to its central role in coagulation, Factor Xa is a primary target for anticoagulant therapies. Drugs like rivaroxaban and apixaban specifically inhibit Factor Xa to prevent excessive clot formation and reduce the risk of thromboembolic events. (Spronk et al., 2014) Moreover, inhibiting Factor Xa can effectively reduce the risk of stroke in patients with atrial fibrillation, which is often caused by blood clots traveling to the brain. In the context of acute coronary syndrome (ACS), where atherosclerotic plaque rupture can lead to clot formation, Factor Xa inhibitors help in reducing the likelihood of recurrent cardiovascular events. (Cocker & Saguil, 2019)

Factor Xa is a viable target for effective anticoagulation. (Spronk et al., 2014) The enzyme is activated by either the intrinsic or extrinsic pathway, leading to the conversion of prothrombin into thrombin, a key event in blood clot formation. Factor Xa's ability to hydrolyze its substrates and facilitate thrombin production makes it a prime target for anticoagulant therapies aimed at preventing thrombosis and managing cardiovascular diseases (CVDs) (Jackson, 2021). The protein structure of Factor Xa (PDB ID: 2P16) obtained from RCSB PDB is utilized in the *In-silico* analysis of this study. Structurally, Factor Xa consists of two subunits—a light chain and a heavy chain—held together by a disulfide bond. The light chain contains epidermal growth factor-like (EGF) domains, while the heavy chain houses the active site, responsible for its proteolytic activity.

Many plants are rich in secondary metabolites, such as polyphenols and flavonoids, which exhibit significant anticoagulant and antiplatelet properties. These compounds can inhibit various enzymes involved in the coagulation cascade, making them promising candidates for developing natural anticoagulants (Lamponi, 2021). Anticoagulant therapy with heparin is associated with several adverse reactions, such as thrombocytopenia, osteoporosis, hypoaldosteronism, and hypersensitivity reactions (Kollias et al., 2020) For this reason, It is essential to establish therapeutic protocols that include the appropriate use and dosage of effective anticoagulant and antithrombotic drugs for both the prevention and treatment of coagulation disorders. This ensures optimal management of clotting abnormalities and reduces the risk of related complications.

Medicinal plants have been recognized as valuable sources of anticoagulant properties. Several plants native to the Philippines possess anticoagulant effects, which are primarily attributed to the presence of bioactive compounds such as flavonoids. These natural substances significantly prevent blood clot formation, offering potential therapeutic benefits for managing clotting disorders. (Sahagun et al., 2021)

Kangkong, scientifically known as *I. aquatica*, is a member of the Convolvulaceae family, commonly found in tropical regions, particularly in Southeast Asia. (Adedokun et al., 2019) It is widely recognized for its nutritional value. Among the abundant and edible green vegetables available in the Philippines, *I. aquatica* has been used in traditional medicine for various health purposes. It is a good source of amino acids, trace elements some other bioactive phytochemicals (Adedokun et al., 2019; Ramzy et al., 2019).

Many studies have already explored its antioxidant activity and anti-inflammatory properties. A study by Roy et al. (2022) explored the bioactive profile of *I. aquatica* and its antioxidant activities. It was revealed that the plant contained high levels of phenolic and flavonoid compounds present in crude and fractionated extracts of *I. aquatica*. This suggests that Kangkong is a good candidate for anticoagulation due to its abundance of phytochemical compounds.

While *I. aquatica* is recognized for its nutritional and medicinal properties, there is a notable absence of focused studies examining its anticoagulant effects, especially regarding its interaction with Factor Xa. Research studies has identified various other plants with established anticoagulant activities, such as *Meriandra dianthera* and *Artemisia dracunculus*, (Duric et al., 2015) which have demonstrated significant effects on coagulation pathways through specific assays like prothrombin time (PT) and activated partial thromboplastin time (aPTT) (Kiflemariam et al., 2022a). These studies highlight the potential for similar investigations into Kangkong. Kangkong shows promise due to its bioactive components, but comprehensive research specifically targeting its anticoagulant effects—especially concerning Factor Xa—remains sparse.

These synthetic anticoagulants such as Heparin and Warfarin have numerous drug interactions that can alter their effectiveness. It requires regular monitoring due to its narrow therapeutic index, which can lead to complications such as bleeding or thrombosis if not managed properly (Bachtel & Israni-Winger, 2020) Plant-based treatments often have a more favorable safety profile compared to synthetic drugs. (Greenwood, 2020) Thus, plant-based treatments are in increased demand.

In vitro and *In silico* analyses are utilized to deeply analyze a comprehensive strategy for validating the anticoagulant properties of natural compounds of *Ipomoea aquatica*. In vitro PT and aPTT Assays directly measure the anticoagulant activity of plant extracts using assays such as Prothrombin Time (PT) and Activated Partial Thromboplastin Time (aPTT). (Félix-Silva et al., 2014) In silico docking analysis will thoroughly predict how well certain compounds will interact with target proteins like Factor Xa. This approach allows researchers to screen large libraries of compounds quickly and identify promising candidates for further experimental validation (Gackowski et al., 2023)

This study aims to investigate the potential of *I. aquatica* extract as a blood-thinning agent for anticoagulation through clotting time using in vitro PT and aPTT assays and explore *I. aquatica* extract potential of inhibiting Factor Xa Hydrolase (PDB ID: 2P16) through molecular docking analysis using AutoDock Vina. Particularly, the objectives of this study are:

1.1. *In vitro* analysis

- **Assess the anti-coagulation properties of Kangkong *(Ipomoea aquatica)* extract through in-vitro PT and APTT Assays.** By measuring these parameters after administering Kangkong *(I. aquatica)* extract, researchers can determine its potential to influence blood clotting.
- **Comparative Analysis of anti-coagulant effects of *Ipomoea aquatica* with those of known anti-coagulants.** To better understand its efficacy and potential applications in clinical settings.
- **Evaluation of the Safety and Potential Side Effects of Kangkong Extract as a Therapeutic Agent.** This is essential for considering its use in both preventive and therapeutic contexts.

1.2. In silico analysis

- Assess the drug-likeness and Toxicity of phytochemical constituents of *I. aquatica* through Lipinski rule of 5 using SWISSADME and ProTox 3.0, respectively.
- Molecular Docking Simulations.
- Binding Affinity Analysis.
- Inhibitory Effects Assessment.

2. Methodology

2.1. PHASE I - In Vitro Analysis

2.1.1. Collection, Preparation, and Ethanolic Extraction of Kangkong (I. Aquatica)

Fresh Kangkong *(I. aquatica)* were obtained locally in Bankerohan Public Market, Agdao Davao City. The plant material was separated and washed with distilled water to remove dust particles. Unlike the roots, which can absorb harmful metals like lead and cadmium, the aerial parts are safer for consumption. (Ashwini, 2017) Hence, only the aerial parts of Kangkong were utilized in making the plant extract.

Figure 1 Air drying of Kangkong (*Ipomoea aquatica*)

The aerial parts of the fresh Kangkong *(I. aquatica)* were air-dried at room temperature (20–22°C) and grinded into a coarse powder. The grinded Kangkong was transferred to an airtight container where Ethanol was then poured into the container until the water spinach was completely submerged. The coarse plant material (200g) was fully submerged in a 1:10 ratio (w/v) of 95% Ethanol Solution (2000 mL) for three days before filtration (Mbbs et al., 2016).

Figure 2 Soaking of Kangkong *(I. aquatica)* in ethanol for 72 hrs

After soaking, the solution was filtered using filter paper and a Buchner funnel to separate the solid materials from the solution. Then, underwent rotary evaporation to ensure the separation of ethanol from the pure plant extract of *I. aquatica* (Walleser, 2019). The final extract was deposited in an airtight container and underwent phytochemical screening. Phytochemical analysis was done to detect the presence of bioactive compounds according to standard methods described by (Aguinaldo et al., 2005 ; Arenas et.al (2017) extracts were subjected to the presence of different phytochemical properties.

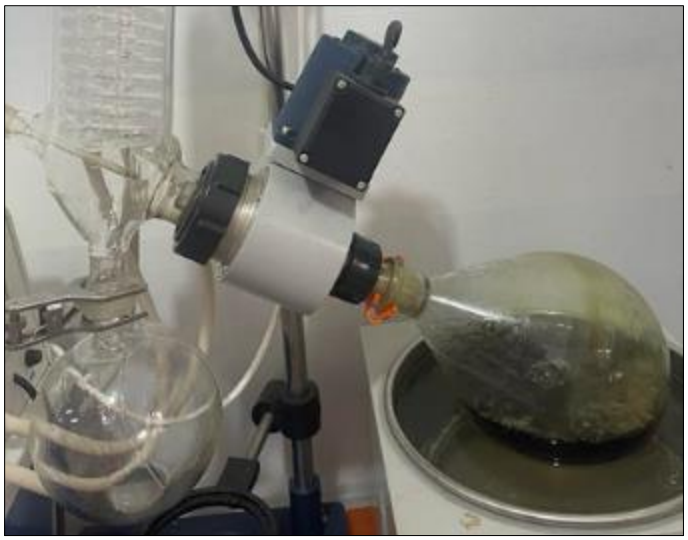

Figure 3 Kangkong-ethanol solution undergoing rotary evaporation

2.1.2. Screening of Donor and Blood Sample Collection

The method was based on the study of (Omar et al., 2017) with minimal modifications. Blood samples were collected via vein puncture from 1 healthy donor (age 18-35 years old). The subject was informed about the study's objective and signed a written informed human participation consent form retrieved from ISEF Forms. (Shafee, et al., 2015)

Inclusion criteria include a healthy donor who has not undergone anticoagulation therapy and other medications, especially antiplatelets, and anticoagulants, in the last 7 days. Donors with medications or supplements were excluded from this study. (Shafee, et al., 2018) The donor is also a non-smoker. Through screening via questionnaire, the donor provided a familial history of cardiovascular diseases and other major coagulopathies. (Manicam et al., 2010).

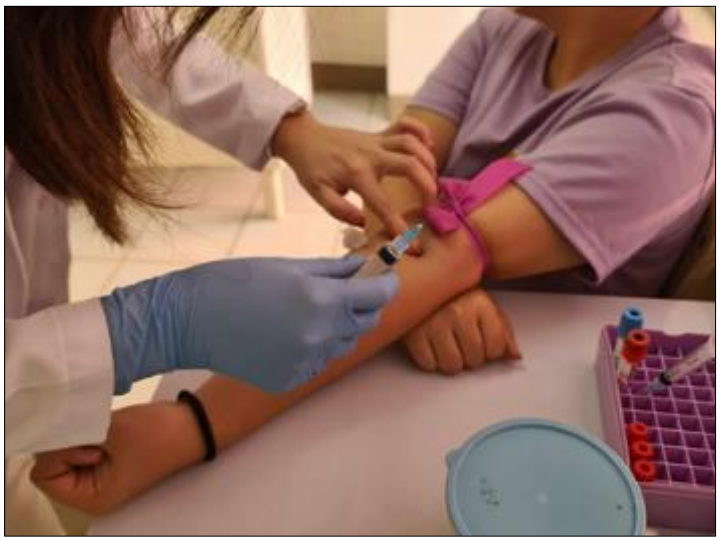

Figure 4 Vein puncture performed by a registered medical technologist at UIC Laboratory

2.1.3. Blood Sample Preparation

Blood samples were drawn via vein puncture 1 healthy donor (age 18-35 years old). The vein puncture was performed by a registered medical technologist in the UIC Clinical Laboratory. The blood is placed separately in containers containing Sodium Citrate to prevent the clotting process.

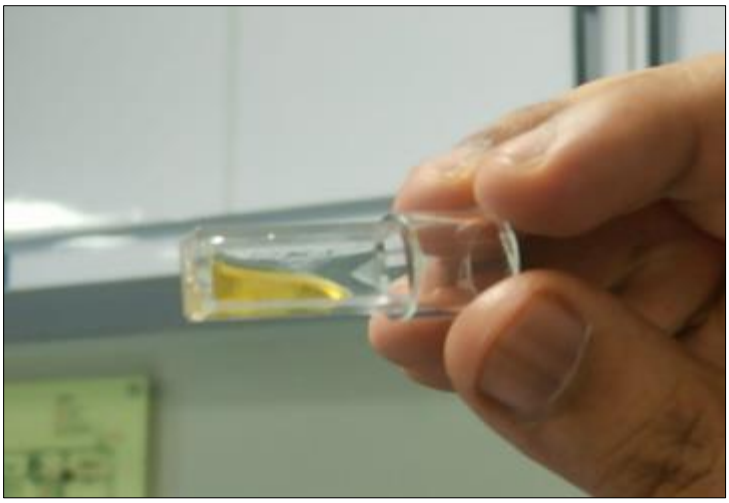

Figure 5 Platelet-poor plasma (PPP) obtained from sodium-citrated blood through centrifugation

Centrifugation (15 minutes at a rate of 3000 rpm) was carried out to separate the blood cells from plasma to obtain platelet-poor plasma (PPP) for the PT and APTT assays. (Chegu, et al., 2018) The tube was then labeled with the participant's name and time of collection. Lastly, the sample was stored in a chiller at -80°C prior to analysis.

2.1.4. In vitro Anticoagulation Assay

The *In-vitro* PT and APTT assays were conducted using a Yumizen G400 Hemostasis analyzer provided by UIC Bonifacio Clinical Laboratory (Corner De Jesus St, Poblacion District, Davao City, 8000 Davao Del Sur). The homeostasis analyzer will automatically provide clotting time which is then recorded for data analysis. Sodium-citrated blood will be used as a negative control (absence of anticoagulant activity) and heparin (1 IU/mL) will be used as a positive control. Each replicate provides 2 results for a total of 6 results for each replication (Omar et al., 2020)

2.1.5. PT Assay

Methods used were based on a study by (Omar et al., 2017) modified anchoring on the UIC Clinical Laboratory machine's instruction manual (Yumizen G400 Hemostasis Analyzer). Prothrombin Time (PT) measures the time it takes for blood to clot through the extrinsic and common coagulation pathways. (MedlinePlus, 2021)

Primarily, equal parts of plasma from normal citrated PPP and Kangkong crude extract were mixed to make a 100 µL plasma-kangkong extract solution. (Dewanjee et al., 2015) The solution was then incubated for 2 mins at 37° C. After 2 mins, a pre-warmed PT reagent was added to the sample.

Table 1 Ratio of samples and PPP in PT Assay

Type of Sample	Amt. of PPP (plasma)	Amt. of sample	Amt. of Reagent
Kangkong extract	25 µL	25 µL	100 µL
Positive Control	25 µL	25 µL	100 µL
Negative Control	50 µL	0 µL	100 µL

Each replicate provides 2 results for a total of 6 results for each replication which were then recorded for analysis.

2.1.6. APTT Assay

According to (MedlinePlus, 2021), activated Partial Thromboplastin Time (aPTT) assesses the intrinsic and common coagulation pathways. The same mixture of plasma-plant extract was utilized in this assay; 50 µL of the plasma-kangkong extract solution was incubated for 10 minutes at 37 °C. then 50 µL of aPTT reagent was added to the mixture, followed by another incubation for 3 mins. Lastly, 50 µL of Sodium Citrate was added to the mixture.

Table 2 Ratio of samples and PPP in the APTT Assay

Type of Sample	Amt of PPP (plasma)	Amt.of sample	Amt. of Reagent	SodiumCitrate Solution
Kangkong extract	25 µL	25 µL	50 µL	50 µL
Positive Control	25 µL	25 µL	50 µL	50 µL
Negative Control	25 µL	0 µL	50 µL	50 µL

Each replicate provides 2 results for a total of 6 results for each replication which were then recorded for analysis.

Figure 6 Conducting PT and aPTT Assay assisted by a registered medical technologist at UIC Laboratory

2.1.7. Statistical Analysis

Statistical analysis was performed using JASP version 0.19.1.0 The values of PT and APTT were compared with the control (heparin). Data were analyzed and reported as median ± standard deviation using one-way ANOVA with post-hoc tests employed to determine if there were significant differences between the plant extract and the control group. A p-value of < .001 is considered statistically significant.

2.2. PHASE II - In-Silico Analysis

2.2.1. Assessment of Drug-Likeness and Toxicity Screening of Phytochemicals from Kangkong (Ipomoea aquatica)

A total of 130 phytochemicals were listed from existing literature of *I. aquatica*. The bioactive compounds identified in the kangkong extract were flavonoids, phenolic, saponins, alkaloids, terpenoids, carbohydrates, metabolites, and essential fatty acids. These literature-based physicochemical compounds underwent druglikeliness screening through SwissADME (http://www.swissadme.ch/), following the Lipinski rule of 5 which states that suggests a compound is more likely to be an effective oral drug if it has no more than five hydrogen bond donors, ten hydrogen bond acceptors, a molecular mass under 500 daltons, and a ClogP not greater than 5. (Neidle, 2012) In drug development, the evaluation of absorption, distribution, metabolism, and excretion (ADME) is being conducted progressively earlier in the discovery phase, when many compounds are under consideration, but physical samples are still limited. (Daina et al., 2017).

Additionally, the phytochemicals also underwent Protox 3.0 Toxicity Screening (https://tox.charite.de/protox3/0) based on the median lethal dose (LD50) values of chemicals, which indicate the amount required to kill 50% of a test population. (Banerjee et al., 2018) ProTox 3.0 was utilized to evaluate the safety profile of these compounds before proceeding through the docking process.

Firstly, 3D Conformers are obtained from online databases through PubChem (https://pubchem.ncbi.nlm.nih.gov/). Then, the SDF Files were changed to PDB Format using PyMOL (Rahman, 2020); The compounds were prepared for docking with MGL AutodockTools (v.1.5.7-latest) and the Autodock Vina software. AutodockTools automatically assigned each ligand's Gasteiger charges and torsional degrees of freedom through Autodock Vina. Once all compounds were optimized, they were saved as PDBQT files for docking.

2.2.2. Preparation of Factor Xa Hydrolase Protein (PDB ID: 2P16) using AutoDock Vina

The 3D structure of the target hydrolase (RCSB PDB: 2P16) is a protein-ligand hydrolase classified as coagulation factor X (EC 3.4.21.6) (Stuart factor) (Stuart-Prower factor). This hydrolase structure has an attached inhibitor APIXABAN (1-(4-methoxyphenyl)-7-oxo-6-(4-(2-oxo-1-piperidinyl) phenyl)-4,5,6,7-tetrahydro-1h-pyrazolo[3,4-c]pyridine-3-carboxamide), an orally bioavailable inhibitor of Factor Xa.

The 3D structure of the FXa hydrolase was downloaded as 3D Model from the RCSB Protein Data Bank (https://www.rcsb.org/) in PDB Format. Following the download, the PDB file underwent energy minimization using the Swiss PDB Viewer (Tuli et al., 2022). It was then imported into MGL AutodockTools for further preparation. Water molecules (HOH302.A - HOH396.A) being used for crystallization and other naturally bounded ligands APIXABAN (GG2.A) and CALCIUM ION CA+ (CA.A), both found on chain A were deleted (Sulimov et al., 2015). Polar Hydrogens and Kollman Charges were added and evenly distributed on the serine protease protein (Sharma et al., 2021). The total residues were reviewed then the modified protein file was saved as a PDBQT file to be accessed by AutoDock Vina.

2.2.3. Manually Generated Receptor Grid Box via CASTp

The active binding residues of the 2P16 FXa Hydrolase were predicted through CASTp (http://sts.bioe.uic.edu/castp/index.). The receptor grid area was determined using AutoDock Vina through MGL AutoDock Tools. With the grid box center aligned to the active binding site, corresponding to the key residues of the receptor protein's main pocket: ILE 16, CYS 42, GLY 43, GLY 44, THR 45, LEU 53, THR 54, ALA 55, ALA 56, HIS 57, CYS 58, GLN 61, THR 98, TYR 99, ASP 102, ILE 103, ALA 104, GLY 136, ILE 137, VAL 138, SER 139, GLY 140, GLY 142, THR 144, VAL 160, TYR 162, PHE 174, PHE 181, ASP 189, ALA 190, CYS 191, GLN 192, GLY 193, ASP 194, SER 195, GLY 196, GLY 197, PRO 198, HIS 199, GLY 211, ILE 212, VAL 213, SER 214, TRP 215, GLY 216, GLY 218, CYS 220, ALA 221, GLY 226, ILE 227, TYR 228, THR229. The binding site was minimized to 50x50x60 Angstrom - 0.375 (Sulimov et al., 2015) to reduce the likelihood of the software generating inaccurate results and irrelevant binding interactions. The XYZ values of the coordinates of the center on the grid box (x: 13.192, y: 47.147, z: 62.735), as well as the dimensions of the grid box (50x50x60), were written in a text document for the configuration file with the exhaustiveness set to level 8 and energy range of 3 as per default protocol.

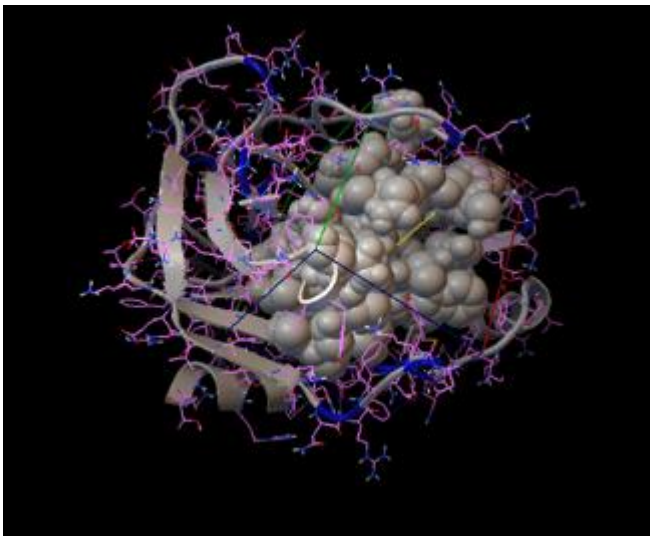

Figure 7 Visualization of the receptor grid box for Factor Xa Hydrolase Protein (PDB: 2P16) using MGL AutoDock Tools v. 1.5.7. Amino acids in the primary binding site, as predicted by CASTp, are displayed as spheres

2.2.4. Molecular Docking Analysis and Simulation via AutoDock

Molecular docking simulations are performed using AutoDock Vina to investigate the binding interactions between the bioactive compounds from *I. aquatica* extract and the selected protein target involved in blood coagulation, which is Factor Xa in Complex with the Inhibitor APIXABAN (PDB ID: 2P16) Docking analysis and simulation were conducted after saving the optimized ligands, protein, and configuration files in the same folder. This process was carried out using the Command Prompt on Windows 10 and 11 systems. Autodock Vina (Trott & Olson, 2010) was employed for docking via the Command Prompt. The working directory was navigated to the folder containing the ligand, protein, and configuration files, followed by executing the command:

"C:\Program Files (x86)\The Scripps Research Institute\Vina\vina.exe" --receptor [protein.pdbqt] --ligand [ligand.pdbqt] --config [config.txt] --log [log.txt] --out [output.pdbqt]"

was written in the prompt for computation (Sharma et al., 2021) The docking was performed, and the binding affinities of the molecules to the Factor Xa Hydrolase (PDB ID: 3D11) were obtained. Each ligand underwent this process three to five times to ensure reliable and accurate results. The output files generated from the simulation included log files in text format and PDBQT files. Upon completion of the computation, the output and protein files (in PDBQT format) were imported into PyMOL for 3D simulation. UCSF Chimera, PyMOL, and Ligplot+ were utilized to visually represent the docking interactions between the proteins and ligands.

2.2.5. Data Scoring and Analysis

The results of the docking computation were saved as a text file in the same folder as the other docking files. This log file outlined the binding affinity levels of various phytocompounds to the proteins. A scoring function assesses the conformation and orientation of the ligand within the protein's binding site, measuring binding affinity, which indicates the strength of the interaction between the ligand and the protein. According to Pantsar and Poso (2018), a more negative binding affinity score suggests a stronger interaction between the ligand and the protein, thereby increasing the likelihood of successful binding. Only the results with the ligand situated inside the main pocket of the protein were considered competent data.

Among the five docking attempts, the output with the lowest binding affinity value was selected as the representative data for the docking interaction of each ligand, provided that the compound was positioned within the protein's main pocket. A lower binding affinity value reflects a stronger interaction between the protein and the ligand (Ali et al., 2018), indicating greater energy release upon binding, which leads to a more stable complex between the molecules (Seo et al., 2021). Consequently, this suggests a higher potential for inhibiting protein-protein interactions.

The docking results are analyzed to rank the ligands based on their binding affinities and to identify the most favorable binding poses for each ligand-protein pair. The interactions are visualized using molecular visualization software using PyMOL and LigPlot+, allowing inspection of key interactions such as hydrogen bonds and hydrophobic contacts. The binding affinities of the bioactive compounds will be compared to those of known anticoagulant drugs, such as heparin to assess their potential efficacy.

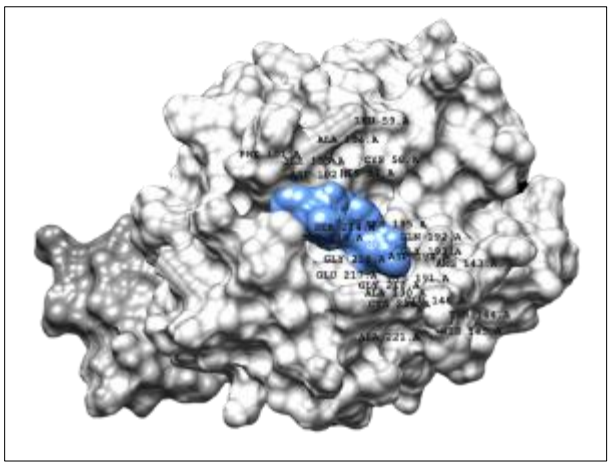

Figure 8 Warfarin (positive control) binding with interacting residues of Factor Xa (PDB ID: 2P16) Hydrolase Protein (blue)

The interacting residues are also evaluated after docking through Ligplot+. The residues of the binding sites of the protein that interact with the ligand will be compared to the residues of the protein that naturally interact with residues of warfarin: CYS 191, TRP 215, ALA 190, ASP 189, GLY 216, CYS 220, TYR 99, GLU 146, GLN 146, VAL 213, SER 195, GLY 218 (Abou-Zied, 2015)

The binding affinity levels were used as bases to rank the ligands to their interaction with the Factor Xa Protein. A descriptive analysis will evaluate the phytocompounds according to their binding affinity levels. Warfarin, an oral anticoagulant commonly used to treat and prevent blood clots (Patel et al., 2022) will be assessed on its docking performance with the Factor Xa Hydrolase Protein. Warfarin can deplete functional vitamin K reserves and thereby

reduce the synthesis of active clotting factors. Binding sites interacting with the interactive molecules were expressed separately per ligand.

3. Results

3.1. In-Vitro Analysis

The present study was conducted to determine if the Kangkong *(I. aquatica)* extract affects the duration of clotting time on a normal-citrated blood sample. PT and APTT assays were used in this study to measure the anti-coagulation activity of Kangkong *(I. aquatica)* extract through its prolonged coagulation time based on the reference value.

PT is utilized to measure the inhibition of extrinsic factors that lead to activation of Factor X while APTT measures the time it takes to inhibit the fibrin clot or the intrinsic pathway. (Wheeler & Gailani, 2016) The platelet-poor plasma (PPP) was treated in three different groups: Crude Kangkong Extract (100% conc.), Positive Control (Heparin), and Negative Control (No Treatment). One-way ANOVA was utilized to determine if significant differences exist between the groups.

Table 3 Clotting Time for PT and APTT Assays

Sample	**PT**	**APTT**
Crude Extract	42.02s ± 2.72s	210s ± 0s
Positive Control	120s ± 0s	210s ± 0s
Negative Control	13.05s ± 0.57s	41.33s ± 3.93s

*Values are expressed as Mean ± S.D. (n=1). aPTT = activated partial thromboplastin time; PT = Prothrombin Time; For the analysis of aPTT, the maximum cut-off time recorded by the coagulation analyzer was 210s. For PT, the maximum was 120s.

In the PT Assay results show that there are significant differences in the clotting time across the three different treatments, $F(2,14) = 8546.06$, $p < .001$. The independent variable accounts for 99.9% of the variance in the clotting time of the treatments. Tukey's HSD was used as a post-hoc test. It reveals that the PPP treated with the Kangkong *(I. aquatica)* Crude Extract ($M = 42.02s$, $s = 2.72$) significantly prolonged the blood clotting time through inhibition of intrinsic factors compared to the negative control ($M = 13.05$, $s = 0.207$), $p < .001$ Moreover, the clinically-used heparin utilized as a positive control ($M = 120.0s$, $s = 0$) displayed a much higher clotting time than the 2 other groups, which is expected for a more potent variable among the three. These findings suggest that the treatment with Kangkong *(I. aquatica)* crude extract significantly prolongs blood clotting time compared to the negative control, indicating that it has anticoagulant properties. Although the extract is not as potent as the clinically used heparin, which had the highest clotting time, it still demonstrates a noticeable effect in delaying clotting.

For APTT Assay, the results also show that there are significant differences in clotting time across the three different treatments in APTT Assay, $F(2,14) = 10012.886$, $p < .001$. Tukey's HSD was also used as a post-hoc test. The test reveals that the Crude Extract ($M = 210s$, $s = 0$) and Positive Control ($M = 210s$, $s = 0$) significantly showed higher inhibition of extrinsic factors than the negative control ($M = 41.333$, $s = 3.93$) Since the machine's limit of measuring was 210 seconds, it was not able to provide exact clotting time beyond the machine's limit. However, these findings suggest that Kangkong *(I. aquatica)* crude extract has significant anticoagulant properties, specifically in inhibiting factors related to the extrinsic pathway of blood coagulation. Both the Kangkong *(I. aquatica)* crude extract and the positive control (Heparin) reached the machine's measurement limit of 210 seconds, indicating that the extract is as effective as the clinically used heparin in prolonging clotting time beyond what the machine could measure.

Furthermore, the statistical analysis of the crude extract relative to the control groups, which are heparin and citrated human plasma highlights the promising anticoagulant properties of Kangkong *(I. aquatica)*. The results are visually presented in Figures 1 and 2. Figure 1 depicts the prolongation of the clotting time of the crude extract compared to the control groups in PT Assay. In Figure 2, the crude extract also shows potential as it can produce the same results with the positive control in APTT Assay, exceeding the machine's limit of recorded clotting time.

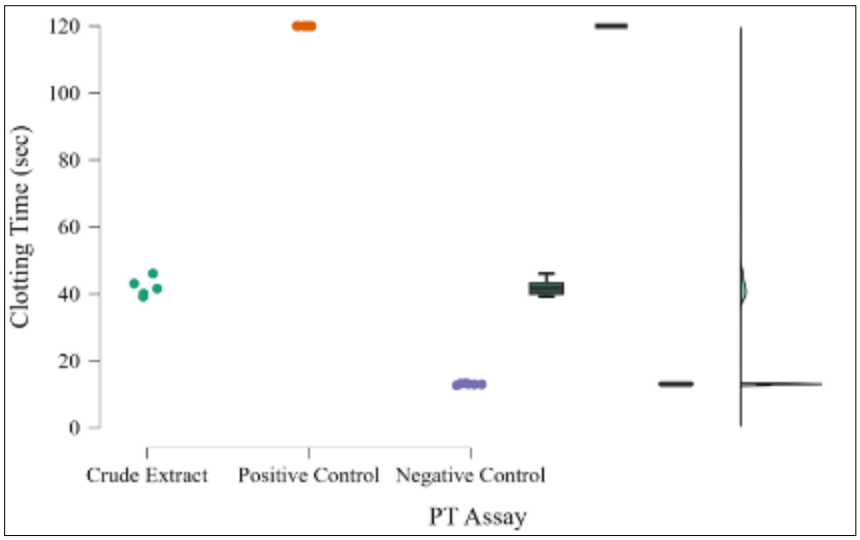

Figure 9 Prothrombin Time (PT) of plasma treated with crude extract of Kangkong *(I. aquatica)* Negative control denotes the normal control plasma without treatments, while Positive Control is the clinically used heparin, the bars represent how dispersed the values are; p < .001 was considered significantly different

4. Discussion

Anticoagulant and procoagulant medications are commonly used to regulate blood clotting in both healthy individuals and those with conditions like cardiovascular disease, diabetes, and bleeding disorders. While numerous drugs have been developed over the years, many are associated with unwanted side effects. As a result, there remains a need for the discovery of new anticoagulant and procoagulant therapies that pose fewer risks. The extrinsic (tissue factors) and intrinsic pathway (contact factors) of the coagulation cascade account to haemostasis. (Omar et al., 2017 ; Nguyen et al., 2002).

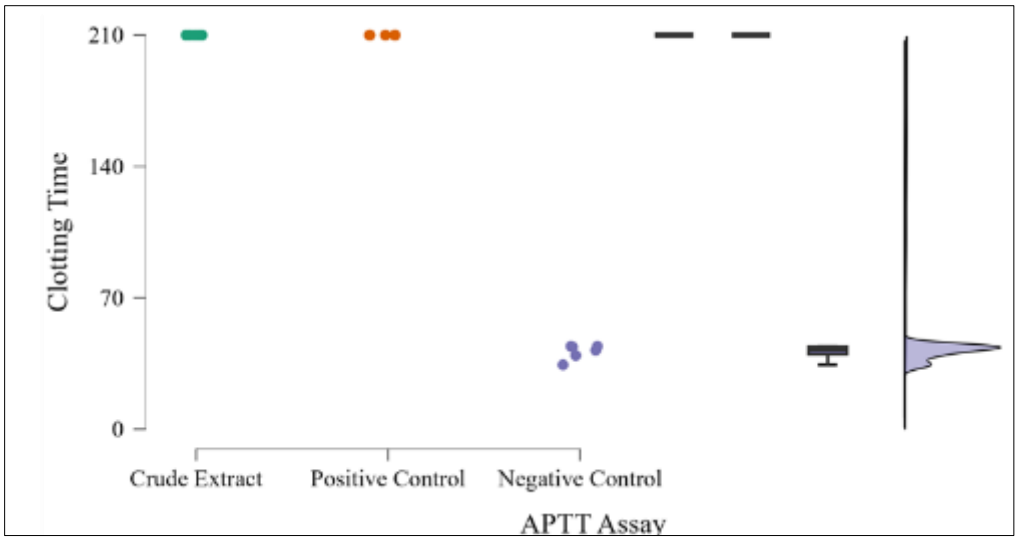

Figure 10 Activated partial thromboplastin time (aPTT) of plasma treated with crude extract of Kangkong *(I. aquatica)*, Negative Control denotes the normal control plasma without treatment (baseline). Heparin was used as a positive control. The bars present how dispersed the values are, p < .001 was considered significantly different

In this study, the anti-coagulation activity of the Kangkong *(I. aquatica)* extract was based on clotting time using PT and aPTT *In vitro* assays. The use of aquatic plants such as aloe camperi, spurges, and latok are able to exhibit anticoagulant properties (Félix-Silva et al., 2014) which makes kangkong a potential candidate for an anticoagulation study.

The standard clotting time for Prothrombin Time (PT) is 10.3s to 14.0s. On the other hand, aPTT's standard clotting time is 34.4s to 46.5s. (Wheeler & Gailani, 2016). Therefore, in comparison to the standard values of PT and aPTT Assay; kangkong extract has displayed its anticoagulant properties and has had coagulation effect on the blood sample tested. *Ipomoea aquatica* prolonged PT at its crude extract for an average of 42.02 seconds. These findings show that the Kangkong *(I. aquatica)* extract has the ability to prolong time. Hence, the extract is able to partially inhibit tissue clotting factors in the extrinsic pathway of the coagulation cascade. (Barmore & Bracken Burns, 2019)

For the aPTT results, the Kangkong *(I. aquatica)* crude extract exceeded the standard reference range of 34.4 to 46.5 seconds, as shown in Figure 10. The extract produced clotting times comparable to the positive control (heparin), even surpassing the machine's measurement limit.

The results of the assays can be compared to a study by Shafee et al. (2018) who studied the anti-coagulation activities of *Malaysian cordata* leaves, where the clotting time of the PPP increased due to the presence of bioactive compounds such as metabolites in the leaves. This suggests that the major bioactive compounds that are also present in Kangkong *(I. aquatica)* such as the flavonoid, alkanoids, and metabolites helped the extract inhibit the intrinsic, extrinsic, and common pathways of the coagulation cascade. (Kangkon Saikia et al., 2023).

Clotting time results of the Kangkong *(I. aquatica)* extract were significantly increased in the PT and aPTT assays conducted. Similarly, a study (Arenajo et al., 2017) evaluated the anticoagulant properties of *Caulerpa lentillifera* crude extract. Results showed significant increases in clotting times, with blood samples treated with the extract exhibiting prolonged coagulation times compared to controls. The findings demonstrated a dose-dependent relationship, indicating that the extract may act as a natural anticoagulant, potentially due to the presence of phytochemicals. (Kiflemariam et al., 2022) These results support the potential therapeutic application of natural extracts in managing coagulation disorders.

In a study by Félix-Silva et al. (2014), the researchers assessed the aqueous leaf extract of *Jatropha gossypiifolia*, finding significant anticoagulant activity in the aPTT test, while no effect was observed in the PT test. The study indicated that the residual aqueous fraction was particularly active, suggesting its potential for therapeutic applications in cardiovascular diseases. The results of this study imply that the increased prothrombin time observed in the PT assay on the kangkong extract offer potential treatment options for cardiovascular diseases.

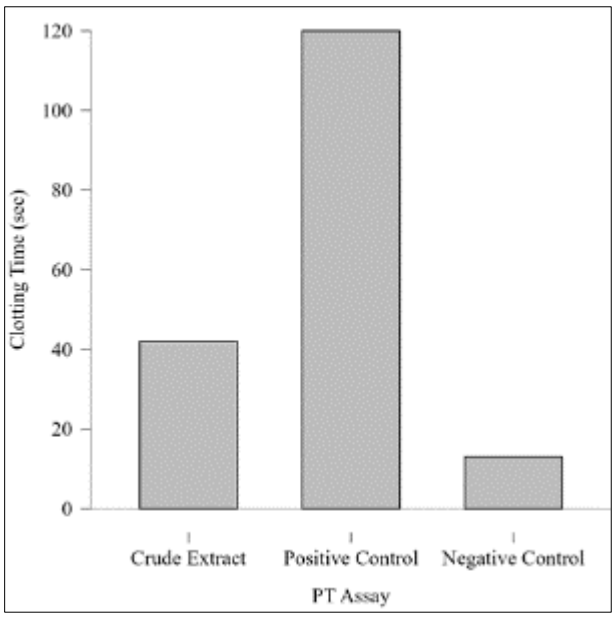

Figure 11 Increased Prothrombin Time (PT) in the assay

Given its capacity to prolong PT and aPTT, Kangkong (*I. aquatica)* extract may provide potential benefits in clinical settings for patients at risk of thrombosis (Ayodele et al., 2019). Additionally, the hepatotoxicity study indicate that *I. aquatica* may impact liver function and related metabolic pathways. Since the liver plays a crucial role in the synthesis of clotting factors, enhanced liver function due to *Ipomoea aquatica* may lead to improved production of coagulation factors, thereby increasing PT and aPTT results.

Heart attacks, strokes, peripheral artery disease (PAD), and angina share a common underlying cause, arterial blockages. These blockages typically develop due to arteriosclerosis, commonly known as the "hardening of the arteries." (Griffin, n.d.) The data obtained in this study align with previous literature, indicating that certain plant extracts exhibit anticoagulant effects by prolonging PT and aPTT.

Inhibiting the extrinsic pathway can reduce thrombus formation associated with atherosclerosis, where plaque rupture can lead to acute coronary events. By targeting this pathway, it may be possible to prevent clots from obstructing blood flow in coronary arteries (Suresh, 2023). Prolonged PT and aPTT suggest that *I. aquatica* may inhibit clotting factors not only in the intrinsic and extrinsic pathways but also in the common pathways of the coagulation cascade. As a result, factors X, V, II, and I could be vulnerable to inhibition. (Omar et al., 2017; Hood & Eby, 2008). Natural anticoagulant agents that inhibit the coagulation process are of greater potential interest for the prevention of CVDs. This study demonstrated that *I. aquatica* extract in pure concentration inhibits clot and thereby increases prothrombin time as illustrated in Fig 11.

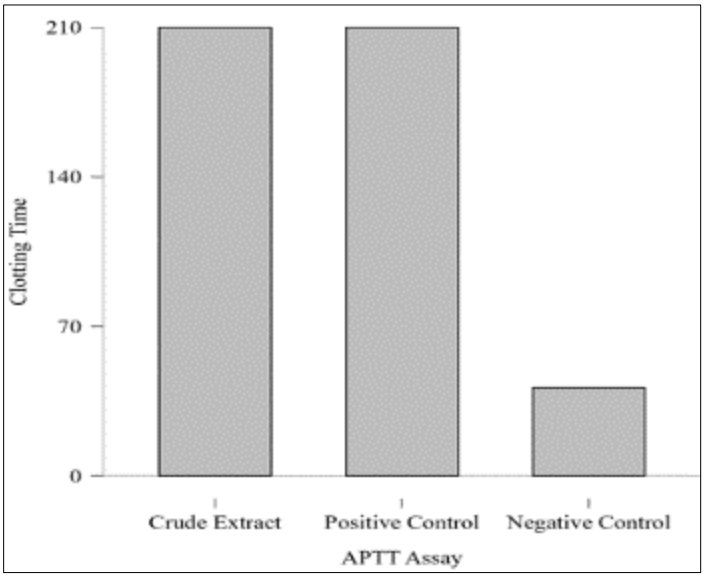

Figure 12 Increased aPTT in the assay

Increased activated partial thromboplastin time (aPTT) indicates a delay in the intrinsic pathway of coagulation, which has significant implications for managing cardiovascular diseases (CVD) and stroke (Grover & Mackman, 2019). Therapies aimed at targeting components of the intrinsic pathway, which have proven effective in inhibiting thrombosis in preclinical studies, are now being evaluated in clinical settings. (Weitz & Chan, 2019) As shown in Fig 12, *I. aquatica* extract has increased APTT results as much as the positive control suggesting successful inhibition of extrinsic pathway in the coagulation cascade. In acute ischemic stroke scenarios, therapies that prolong aPTT can be beneficial. They may be used alongside thrombolytic treatments to prevent secondary thrombus formation during reperfusion therapy, thus improving overall outcomes. (Furie, 2019)

Overall, the *In-vitro* results in this study show the effective inhibition of intrinsic and extrinsic pathways through *I. aquatica* extract anticoagulant activity. The findings provide valuable insights that can guide future research on combating CVDs such as ischemic stroke and coronary artery blockages.

4.1. In-Silico Analysis

This study aimed to determine and analyze the 130 primary phytochemicals found in Kangkong *(I. aquatica)* targeting Factor Xa Hydrolase active binding sites that initiates the production of fibrin and thrombin which is a major factor in both intrinsic and extrinsic pathway in the coagulation cascade. The 130 were subjected to SwissADME and Protox 3.0 Screening, following the Lipinski rule of 5 and Predicted Toxicity Class, respectively. Only 70 were found to have passed both screening.

Table 4 Phytochemicals found in *I. aquatica*

a	b	c	d	e	f	g
3-Carene	136.23	0	0	4.29	0	5
Benzene, 1,3-bis(1,1-dimethylethyl)	190.32	0	0	5.58	1	5
2-(7-t-Butoxy-heptyl)-5-methoxy-cyclopent-2-enone	282.42	3	0	2.24	0	5
Glutamine	146.14	3	4	-3.58	0	6
Glucose	180.16	5	6	-2.75	0	6
Nicotinic acid	123.11	1	3	-1.3	0	5
Riboflavin	376.36	5	8	-0.54	0	6
Vitamin B1	300.81	2	3	0.32	0	4
Vitamin C	176.12	4	6	-2.60	0	5
Anthocyanins	287.24	5	6	0.32	0	5
Isorhamnetin	316.26	4	7	-0.31	0	5
Tamarixetin	316.26	4	7	-0.31	0	5
Luteolin	286.24	4	6	-0.03	0	5
Apigenin	270.24	3	5	0.52	0	5
Kaempferol	286.24	4	6	-0.03	0	5
1-hexadecanoylpyrrolidin	323.51	0	2	4.16	1	4
Gallic Acid	170.12	4	5	-0.16	0	4
Cianidanol	290.27	5	6	0.24	0	6
Vanillic Acid	168.15	2	4	0.74	0	4
Caffeic Acid	180.16	3	4	0.70	0	5
P-coumaric acid	164.16	2	3	1.28	0	5
Ellagic acid	302.19	4	8	0.14	0	4
N-(14-Methylhexadecanoyl)pyrrolidine	323.56	0	1	4.56	1	4
Aspartic acid	133.10	3	5	-3.59	0	4
Threonine	119.12	3	4	-3.46	0	6
Glutamic acid	147.13	3	5	-3.18	0	5
Proline	115.13	2	3	-2.59	0	4
Glycine	75.07	2	3	-3.58	0	5
Tyrosine	181.19	3	4	-1.70	0	4
Lysine	146.19	3	4	-2.67	0	4
Histidine	155.15	3	4	-3.74	0	6
Arginine	174.20	4	4	-3.28	0	6
Fructose	180.16	5	6	-2.75	0	6
Malic Acid	134.09	3	5	0	0	5
Oxalic Acid	90.03	2	4	-1.51	0	4

Feruloyl-2-hydroxyputrescine	280.32	4	5	0.06	0	4
3-Hydroxycoumarin	162.14	1	3	1.04	0	4
Chlorogenic Acid	354.31	6	9	-1.05	0	5
4-Hydroxybenzoic acid	138.12	2	3	0.99	0	5
Sinapic Acid	224.21	2	5	0.73	0	4
Ferulic Acid	194.18	2	4	-1.00	0	4
Acrylic Acid	72.06	1	2	-0.08	0	5
Phenylacetic Acid	136.15	1	2	1.66	0	5
Succinic Acid	118.09	2	4	-0.54	0	5
Glutamic Acid	147.13	3	5	-3.18	0	5
Dihydroxybutanoic Acid	120.10	3	4	-1.25	0	5
Phthalic Acid	116.13	2	4	1.20	0	5
Fumaric Acid	116.07	2	4	-0.64	0	4
9-Decenoic Acid	170.25	1	2	2.47	0	5
Arachidic Acid	312.53	1	2	5.13	1	4
Feruloyl2-hydroxy putrescin-e	313.35	3	4	1.89	0	4
3-Hydroxycoumarin	162.14	1	3	1.04	0	4
1-Nonadecane	424.40	3	10	0.06	0	6
cis-4-Decenedioic Acid	200.23	2	4	1.46	0	4
Palmitic Acid	256.42	1	2	4.19	1	4
Lauric Acid	200.32	1	2	3.15	0	4
L-Threonine	119.12	3	4	-3.46	0	6
L-Homoserine	119.12	3	4	-3.46	0	5
L-Fucitol	166.17	5	5	-1.95	0	6
Pentopyrase	120.1	3	4	-1,25	0	6
Xylitol	313.3	3	4	1.89	0	6
D-Galactofuranose	180.16	5	6	-2.75	0	6
Tetraethylene Glycol	194.23	2	5	-1.27	0	5
Octopamine	153.18	3	3	0.33	0	4
Diglycerol	166.17	4	5	-1.95	0	5
Erythritol	122.12	4	4	-1.91	0	6
Linalool	154.25	1	1	2.59	0	5
Thioacetic Acid	74.12	0	1	-0.37	0	4
N-trans-feruloyltyramine	313.35	3	4	1.89	0	4

a = Ligands; **b** = Molecular Weight (g/mol, <**500 Da**); **c** = Number of Hydrogen bond donors (<**5**); **d** = Number of Hydrogen bond acceptors (<**10**); **e** = M Log $P_o/_{vv}$ (**≤4.15**); **f** = Number of Violations (<**1**); g = Tox Prediction (**>4) g** = Predicted Toxicity Class

The table presented the physicochemical properties of the 70 ligands as per evaluation of SwissADME and Protox 3.0. 3-Carene, Benzene, Heptadecane, Sulfurous acid, Cycloheptasiloxane,ntetradecamethyl,Cyclooctasiloxane, hexadecamethyl-, Sulfurous acid, cyclohexylmethyl tetradecyl ester, 1-hexadecanoylpyrrolidine,N-(14-

Methylhexadecanoyl)pyrrolidine, Chlorogenic Acid, Palmitic acid, Apigenine, and Nonadecane had one violations. However, these violations were still considered acceptable , the overall drug-like characteristics of the ligands were anyhow accepted.

4.2. Scoring Functions

The docking results are analyzed to rank the ligands according to their binding affinities and to determine the most favorable binding poses for each ligand-protein pair. The interactions are visualized using molecular visualization software, specifically PyMOL among the five docking attempts that occupied the binding pocket. A standardized -7 affinity value was given to pinpoint among the 70 phytochemicals that passed having a higher chance of interacting with the 2p16 protein. It was then narrowed down to 10 phytochemicals. Each ligand conformation (1st pose) was then integrated to PDB files for visualization.

Table 5 10 Phytochemicals with -7.0 and Lower Binding Affinity Values

a	**b**	**c**	**d**
Tamarixetin	5281699	-8.7	output 1
Kaempferol	5280863	-8.6	output 5
Isorhamnetin	5281654	-8.5	output 3
Chlorogenic acid	1794427	-8.4	output 4
Anthocyanin	128861	-8.3	output 1
Cianidanol	9064	-8.2	output 2
Apigenin	5280443	-7.8	output 2
Riboflavin	493570	-7.3	output 1
N-Trans-feruloyltramine	5280537	-7.1	output 5
Ellagic acid	5281855	-7.0	output 4

a = phytochemical name; **b** = CID from PUBCHEM (<**5**); **c** = binding affinity values; **d** = output number

Among all the phytochemicals with -7.0 and Lower Binding Affinity values, the lowest binding affinity score was Tamarixetin 4-methoxy quercetin, followed by Kaempferol and Isorhamnetin 3′-methoxy quercetin, Chlorogenic acid, Anthocyanins and Cianidanol are the lead based on our ranking. The ligands displayed a wide range of impressive binding affinities, in which Tamarixetin exhibited the lowest score of -8.7 kcal/mol, closely followed by Isorhamnetin 3′-methoxy quercetin and Kaempferol with -8.6 to -8.5 kcal/mol. While the other ligands including Chlorogenic acid, Anthocyanin, Cianidanol, Apigenin, Riboflavin, N-Trans-feruloyltramine and Ellagic acid had a binding affinity that varied from -8.4 and -7.0 kcal/mol. The insights gained from this data are highly valuable in furthering the study of these ligands as potential inhibitors, enhancing our understanding of their therapeutic potential for treating cardiovascular diseases. The assessment of each compound's binding affinities, along with the identification of interactive residues like hydrogen bonds and hydrophobic interactions, was essential in determining the inhibitory potential of each compound. In molecular docking, binding affinity reflects the strength of the interaction at a specific binding site between a ligand and a receptor, predicting both the likelihood and intensity of their association. Particularly, the significant role of hydrogen bonds and hydrophobic interactions between molecules greatly influences the effectiveness of the phytochemicals from *I. aquatica* binding to FXa hydrolase protein. Its primary goal is to rank ligands based on their binding affinity. These are essentials in helping to identify potential drug candidates; docking generally provides excellent results in finding the correct ligand pose, showing strong agreement with the crystal structures of the corresponding protein-ligand complexes (Spassov, 2024).

4.3. Protein and Ligand Interactions between interacting residues

Blood Coagulation plays a big role in causing heart disease, stroke, and other cardiovascular complications. The target hydrolase human activated coagulation factor X protein complexed with apixaban inhibitor, (RCSB PDB: 2P16) binding sites plays a crucial role in initiating the production of fibrin and thrombin which is a major factor in both intrinsic and extrinsic pathway in the coagulation cascade, stimulating multiple intracellular signaling pathways through G-protein-coupled protease-activated receptors (PARs). Stable interactions of these receptors and the factor X protein take place between the FVIIa 140s loop and the β-strand B2 region of FXa (Venkateswarlu et al., 2003), which is close to the

sodium-binding domain, as well as between the 160s loop and the N-terminal activation loop regions, thereby engaging the protein's largest binding pocket, measuring 158.486 $Å^3$. Key residues within the Factor Xa in Complex with the Inhibitor Apixaban, namely THR98, TYR99, PHE174, ASP189, ALA190, CYS191, GLN192, TRP215, GLY216 and TYR228 are responsible for the interaction with the Inhibitor Apixaban (Alexander, 2007).

This comprehensive molecular docking study examined the interactions between 10 phytochemicals docked with the active binding site of Factor Xa. The study focused on the interaction of these phytochemicals with the Factor Xa hydrolase binding residues, which are situated in and around the central pocket of the Factor Xa Hydrolase protein. The findings showed that all 10 ligands, in their highest predicted conformations, formed at least one interaction with key amino acid residues in the active binding site. Among the various ligands, the binding affinities of the six most notable compounds were above -8.0 kcal/mol. In addition to demonstrating strong binding, these ligands interacted with the FXa hydrolase protein.

Table 6 Ligplot Analysis of the 10 Phytochemicals

a	**b**	**c**
Tamarixetin	ASP189(A) [2.80], SER195(A) [2.64, 2.70], GLU146(A) [2.76]	SER214(A) CYS191(A) ALA190(A) GLY216(A) GLY226(A) TRP215(A) VAL213(A) GLY218(A) CYS220(A) GLU192(A) ASP189(A) GLU146(A)
Kaempferol	SER195(A) [2.73, 2.56], ASP189(A) [2.83], GLN192(A) [2.79]	GLY226(A) VAL213(A) CYS220(A) SER214(A) CYS191(A) TRP215(A) GLY216(A) ALA190(A) GLY218(A) GLU146(A) SER195(A) ASP189(A) GLN192(A)
Isorhamnetin		LYS96(A) THR98(A) GLU97(A) TRP215(A) TYR99(A) GLY216(A) HIS57(A) GLN192(A)
Chlorogenic acid	GLN192(A) [2.72, 3.11], GLU97(A) [2.63]	GLN192(A) CYS191(A) GLY216(A)

		TRP215(A) TYR99(A) PHE174(A) THR98(A) GLU97(A)
Anthocyanin	SER195(A) [2.65], ASP189(A) [2.73], ARG143(A) [3.04], GLU146(A) [2.88]	VAL213(A) GLY226(A) CYS191(A) SER214(A) SER195(A) ASP189(A) TRP215(A) ALA190(A) GLY218(A) GLY216(A) ARG143(A) GLN192(A) GLU146(A) CYS220(A)
Cianidanol	ASP189(A) [2.77], SER195(A) [2.68], GLN192(A) [2.70], GLU146(A) [2.97]	ALA190(A) SER214(A) GLY226(A) ASP189(A) CYS191(A) SER195(A) VAL213(A) TRP215(A) CYS220(A) GLY216(A) GLN192(A) GLY218(A) GLU146(A)
Apigenin	LEU235(A) [3.07], LEU47(A) [3.09]	ASP239(A) GLU124(A) LEU235(A) PHE99(L) LEU123(A) ARG113(L) ALA112(L) LEU47(A) CYS132(L)
Riboflavin	TYR130(L) [2.92], GLY114(L) [2.98], CYS132(L) [2.99], GLY128(L) [3.00],	TYR130(L) GLY114(L) CYS132(L) PRO120(A) GLY128(L) THR127(L) ARG113(L) PRO131(L) LEU47(A)

	THR127(L) [2.95]	SER48(A) GLU49(A) PHE114(A)
N-Trans-feruloyltyramine	GLU97(A) [3.10], ARG143(A) [3.05], GLU146(A) [2.79]	GLU97(A) LYS96(A) THR98(A) TYR99(A) PHE174(A) TRP215(A) GLY216(A) GLY218(A) GLN192(A) CYS191(A) ARG143(A) GLU146(A)
Ellagic acid	LYS230(A) [3.14, 2.84], ARG165(A) [2.91], VAL163(A) [2.74, 2.84], CYS182(A) [3.07]	LYS230(A)MET131(B) PHE181(A) THR132(A) ARG165(A) TYR162(A) VAL163(A) CYS182(A)

a = phytochemical name; **b** = hydrogen bonds with interacting residues; **c** = Hydrophobic interactions with interacting residues; **bold** = binding sites of FXa hydrolase

Tamarixetin, Kaempferol, Isorhamnetin, Chlorogenic acid, Anthocyanin, and Cianidanol emerged as the top ligands in this ranking. The ligands displayed a wide range of impressive binding affinities, with Tamarixetin showing the highest value at -8.7 kcal/mol, closely followed by Kaempferol at -8.6 kcal/mol.

The binding affinities of the remaining ligands, including Isorhamnetin, Chlorogenic acid, Anthocyanin and Cianidol varied between -8.5 and -8.2 kcal/mol. The consistent outcomes underscore the potency of the compounds' binding activity and their specific interaction with critical residues implicated by the factor X protein complexed with apixaban inhibitor host cell binding process. The insights gained from this data are highly valuable for advancing the investigation of these ligands as potential anticoagulant agents, thereby enhancing our understanding of their therapeutic potential in relation to blood clotting disorders and cardiovascular diseases.

The evaluation of each compound's inhibitory potential was based on analyzing their binding affinity levels and the involvement of interactive residues, including hydrogen bonds and hydrophobic interactions between the molecules. The binding affinity within the molecular docking indicates the validity of the interaction occurring at a specific binding site between ligand and a receptor, thereby effectively blocking thrombus formation and inhibiting thrombin generation in a concentration-dependent manner.

These compounds show promise as anticoagulants. Activated factor Xa, which plays a crucial role in the blood coagulation pathway, is an appealing target for the development of anticoagulant drugs (Gackowski et al., 2023).

4.4. Top Three Phytochemicals with the highest binding affinity and prominent interacting residues

Tamarixetin demonstrated interaction with ten crucial binding sites on the hydrolase FXa protein through G-protein-coupled protease-activated receptors (PARs) which takes place between the FVIIa 140s loop and the β-strand B2 region of FXa—specifically, SER214(A), CYS191(A), ALA190(A), GLY216(A), GLY226(A), TRP215(A), VAL213(A), GLY218(A), CYS220(A) and GLU192(A) —engaging in hydrophobic interactions with all ten of these interacting residues. It interacted with 5 notable amino acids within the hydrolase FXa protein within the 10 key residues within the Factor Xa in Complex with the Inhibitor Apixaban. Precisely, it established two moderate hydrogen bonds with ASP189(A), SER195(A) and GLU146(A) at distances of 2.80 Ω, 2.64 Ω and 2.70 Ω, and 2.76 Ω respectively.

Demonstrating a collective binding affinity of -8.6 kcal/mol, Kaempferol is the second most influential compound in regards to binding affinity among the ligands tested. This ligand exhibits a strong potential for interaction with the FXa protein, highlighting their promising inhibitory capabilities. The crucial binding sites of FXa protein—specifically GLY226(A), VAL213(A), SER214(A), CYS191(A), TRP215(A), GLY216(A), ALA190(A), GLY218(A), SER195(A), ASP189(A) and GLN192(A), has been pinpointed as an interacting residue specifically for Kaempferol, fostering a hydrophobic interaction of 2.73 and 2.56 Å, 2.83 Å, and 2.79 Å with the SER195(A), ASP189(A) and GLN(A) amino acids respectively, which are key residues within the Factor Xa in Complex with the Inhibitor Apixaban.

Isorhamnetin interacts with crucial binding sites—THR98(A), TRP215(A), TYR99(A), GLY216(A), HIS57(A) and GLN192(A)—on the FXa protein, which binds through G-protein-coupled protease-activated receptors (PARs). It exhibits a binding affinity of -8.5 kcal/mol, showing significant potential for effectively preventing thrombus formation and inhibiting thrombin generation in a manner that depends on the concentration. Although its binding strength is slightly lower, the compound's interactions with key residues within the Factor Xa combined with its hydrophobic nature, make it a promising candidate for an anticoagulation agent.

4.5. Other Phytochemicals with notable interacting residues

Chlorogenic acid interacted with key residues within the Factor Xa binding sites—GLN192(A), CYS191(A), GLY216(A), TRP215(A), TYR99(A), PHE174(A) and THR98(A)—crucial for preventing thrombus formation and thrombin generation in a concentration-dependent manner. Moderate hydrogen bonding (2.72 and 3.11 Å and 2.63 Å) with GLN192(A) and GLU97(A) and hydrophobic contact with GLN192(A), CYS191(A), GLY216(A), TRP215(A0, TYR99(A), PHE174(A), THR98(A) and GLU(A) displayed a binding affinity of -8.4 kcal/mol. These hydrogen bonds enhance the ligand's specificity for receptor binding, while simultaneous hydrophobic interactions strengthen its overall stability. Based on the study of Wang et al. (2017), Chlorogenic acid was found to delay activated partial thromboplastin time, prothrombin time, and thrombin time, which makes it a strong anticoagulant agent for future development of anticoagulant medications.

Anthocyanin showed a binding affinity of -8.3 kcal/mol, interacting with ten important binding sites: VAL213(A), GLY226(A), CYS191(A), SER214(A), TRP215(A), ALA190(A), GLY218(A), GLY216(A), GLN192(A), and CYS220(A). It also formed hydrogen bonds with the residues SER195(A) and ASP189(A), measured at distances of 2.65 Å and 2.73 Å.

On the other hand, Cianidanol exhibited hydrophobic interactions with several residues, including ALA190(A), SER214(A), GLY226(A), CYS191(A), VAL213(A), TRP215(A), CYS220(A), GLY216(A), and GLY218(A). Additionally, it established hydrogen bonds at distances of 2.77 Å, 2.68 Å, and 2.70 Å with ASP189(A), SER195(A), and GLN192(A), respectively. This highlights its hydrophobic interactions and a strong binding affinity of -8.2 kcal/mol.

4.6. Visualization of the top three Phytochemicals with the most interacting residues

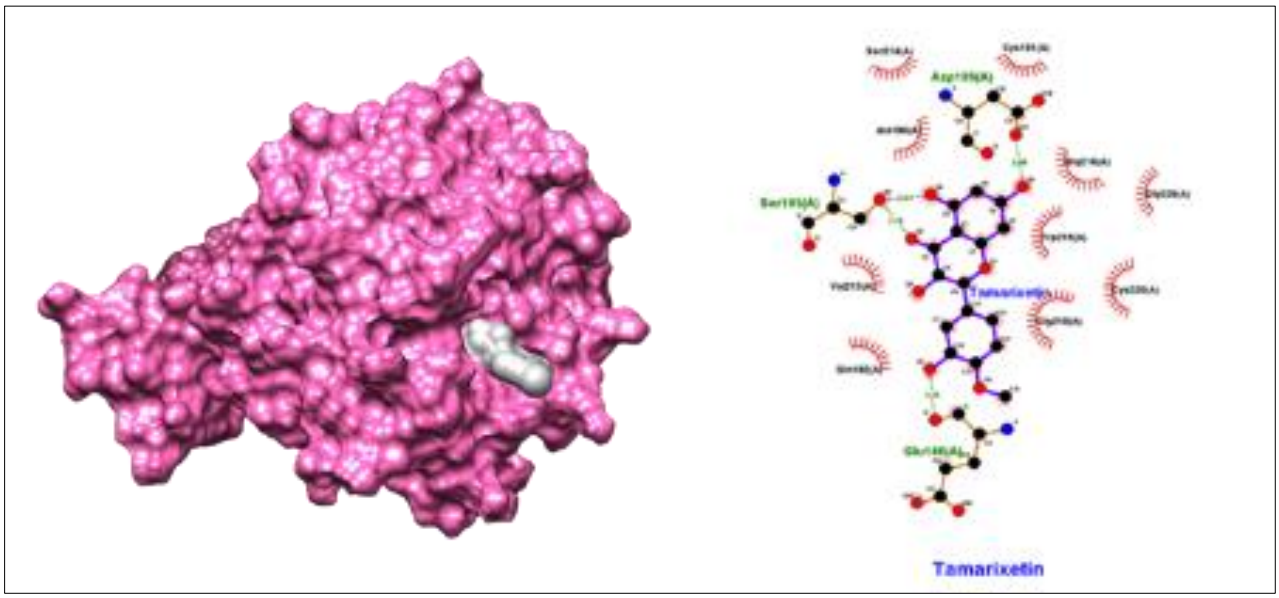

Figure 13 Tamarixetin-Factor Xa complex. Chimera 3D visualization (left); Ligplot+ hydrogen bonds and hydrophobic interactions 2D visualizations (right)

The four remaining ligands, after the top six that exceeded -7.0 kcal/mol, included Apigenin, which had a binding affinity of -7.8 kcal/mol, followed by Riboflavin, N-Trans-feruloyltyramine, and Ellagic acid, with respective binding affinities of -7.3 kcal/mol, -7.1 kcal/mol, and -7.0 kcal/mol. Both N-Trans-feruloyltyramine and Ellagic acid were notable for their interactions with residues THR98(A), TYR99(A), PHE174(A), TRP215(A), GLY216(A), GLY218(A), GLN192(A), CYS191(A), and PHE181(A), highlighting their hydrophobic interactions with these key residue.

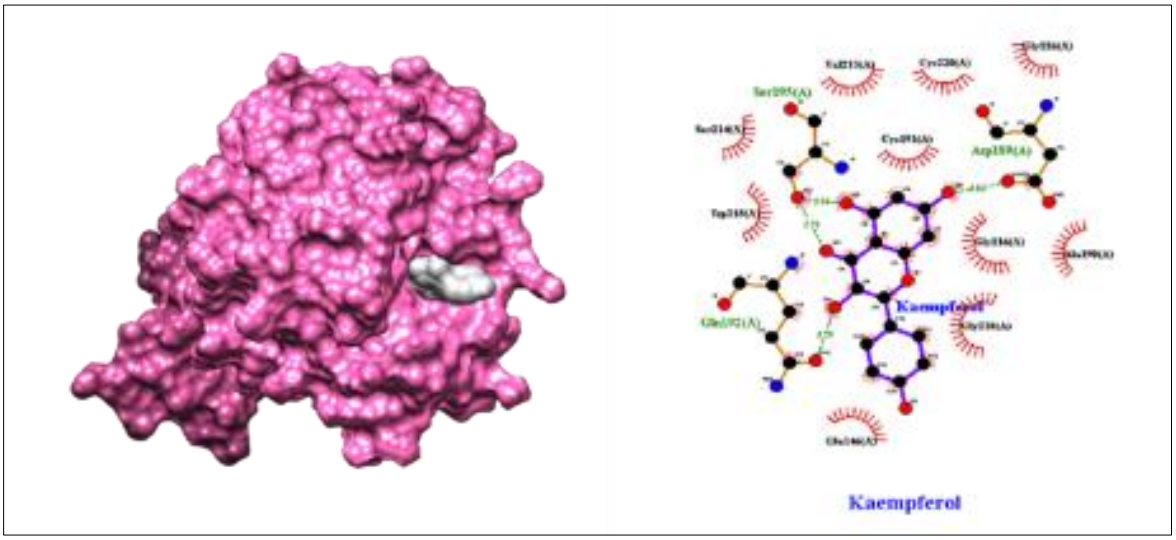

Figure 14 Kaempferol-Factor Xa complex. Chimera 3D visualization (left); Ligplot+ hydrogen bonds and hydrophobic interactions 2D visualizations (right)

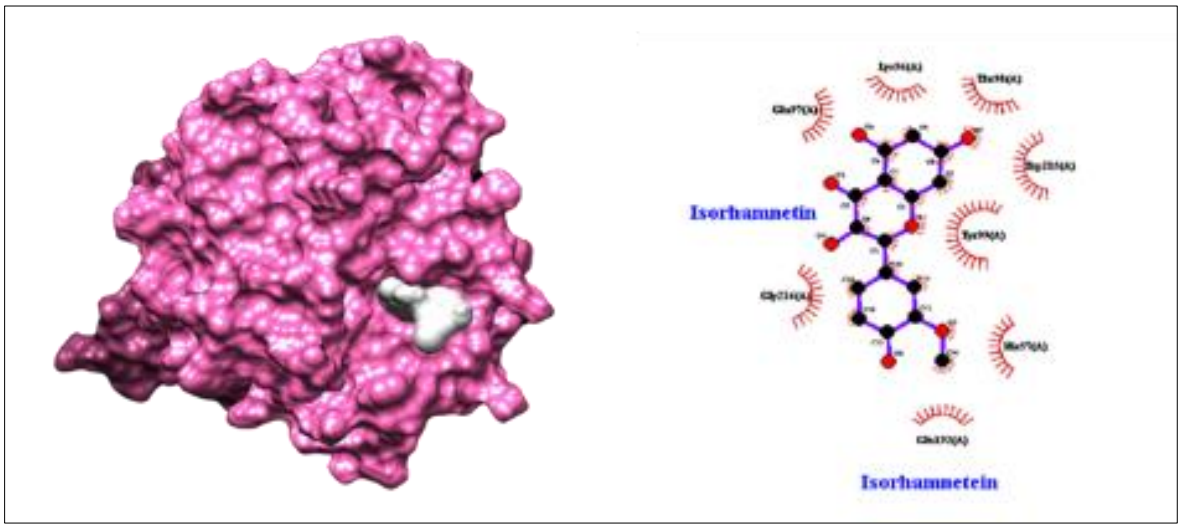

Figure 15 Isorhamnetin-Factor Xa complex. Chimera 3D visualization (left); Ligplot+ hydrogen bonds and hydrophobic interactions 2D visualizations (right)

5. Conclusion and Recommendations

Significant findings were obtained from the *In vitro* and *In silico* docking analyses of *I. aquatica* extract, highlighting its potential as a blood-thinning agent for anticoagulation by inhibiting Factor Xa Hydrolase (PDB ID: 2P16) in response to cardiovascular diseases. This study was conducted to evaluate the effect of Kangkong *(I. aquatica)* extract on the clotting time of normal citrated blood samples.

From the *in vitro* analysis, the researchers explored the anticoagulant properties of Kangkong *(I. aquatica)* extract by examining its effects on clotting time through *In vitro* assays using prothrombin time (PT) and activated partial

thromboplastin time (aPTT). The researchers found that the crude extract significantly prolonged PT and aPTT results, exceeding the standard reference range (34.4 to 46.5 seconds), surpassing the measurement limits of the machine we used compared to those of the positive control (heparin). This finding suggests that Kangkong *(I. aquatica)* has the potential to delay clotting by partially inhibiting the tissue clotting factors involved in the extrinsic pathway of the coagulation cascade. These results highlight the extract's promising potential as a natural anticoagulant. To achieve more accurate results, it is recommended to adjust the heparin ratio used in the machine (ie. 40 µL blood, 40 µL heparin). This modification will enhance the precision of readings and improve the reliability of the data collected in our study.

To improve the accuracy and reliability of our research findings, it is essential to increase the number of blood donors participating in the study. A larger and more diverse donor pool will enhance the representativeness of the data, allowing for more precise assessments of the anticoagulant properties being investigated. By including samples from various demographics, we can better understand how factors like age, ethnicity, and health status may influence results. Additionally, a larger sample size will reduce variability and strengthen the statistical power of our analyses, leading to more robust conclusions and effective clinical applications.

Conducting a comprehensive safety profile is a critical step in the anticoagulation drug development process, involving an in-depth assessment of off-target interactions, cytotoxicity, and any potential adverse effects associated with the compound. This evaluation is essential to ensure both the safety and efficacy of the drug before advancing to clinical trials. Furthermore, performing studies with animal models is advisable, as these investigations provide valuable insights into the compound's pharmacokinetics, including its absorption, distribution, metabolism, and excretion within the body. Gaining an understanding of the compound's biodistribution also sheds light on how it interacts with various tissues and organs in a living organism. By synthesizing findings from both safety profiling and animal studies, researchers can achieve a clearer understanding of the extract's overall anticoagulation efficacy and its potential impact within a complex biological system. This comprehensive approach lays a strong foundation for informed decision-making in the subsequent stages of drug development.

Conversely, the *In silico* docking analysis of the anticoagulant properties of Kangkong *(I. aquatica)* extract, specifically targeting Factor Xa Hydrolase (PDB ID: 2P16), revealed that among the 10 docked and analyzed phytochemicals, Tamarixetin exhibited the strongest binding affinity for the FXa hydrolase protein, thereby achieving the highest rank. However, the findings suggest that all 10 docked phytochemicals from I. aquatica have the potential to act as FXa hydrolase inhibitors in an in-silico setting.

Future investigations should focus on dose-response studies to identify the most effective inhibitory concentrations, ensuring the safe development and use of *I. aquatica* as a potent anticoagulant agent for upcoming medications. Moreover, incorporating molecular techniques such as biochemical assays or molecular dynamics simulations can deepen our understanding of the specific interactions, binding kinetics, and structural changes that Kangkong-derived phytochemicals induce on the FXa hydrolase protein. This holistic approach will enhance our insights into the compound's mechanisms of action and support the development of effective anticoagulant therapies.

In conclusion, while the *In vitro and In silico* docking analyses provide promising preliminary results, the recommendations highlighted above underscore the importance of conducting thorough experimental validation, safety assessments, and further mechanistic investigations. These steps are essential for advancing the development of anticoagulant medications aimed at treating cardiovascular diseases.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of informed consent

The participant is informed on how their blood sample will be used. Participants had understood the implications of the research. The confidentiality of the participants' data must remain hidden. The researchers have assessed any potential risks that will result in any possible dangers relating to the research. A consent form is signed and read by the participant.

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