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Original Article

Multidimensional assessment of natural ligands fisetin, morin and rutin against factor XIIa activity using *in vitro* and *in silico* studies

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Abstract

The discovery of selective inhibitors of factor XIIa (FXIIa) is an attractive approach for development of new antithrombotics that do not interfere with normal hemostasis. Here we report an *in vitro* chromogenic assay and *in silico* molecular modeling-based integrated protocol for predicting the inhibitory aptitude of natural flavonoids, Fisetin, Morin, Rutin and the synthetic derivative (FXIIa-IN-4) of the FXIIa enzyme. The frontier molecular orbitals (FMO) analysis, accompanied by the electrostatic potential (ESP) maps and a non-covalent interaction (NCI) map, provided the information about electronic reactivity of the title compounds. Chromogenic assays data of assessed flavonoids, Fisetin, Morin and Rutin have been shown a dose-dependent inhibition of FXIIa activity, in which at higher concentration (1000 µM) they exhibited about 52.6%, 57.1% and 71.9% inhibition, respectively. Molecular docking studies showed that Rutin has the lowest binding energy with FXIIa (8.6 kcal/mol) and Fisetin has with optimal balance between affinity, structural compactness and reactivity. In addition, molecular dynamics enables comparison of the stability and flexibility of the various ligand–protein complexes. Fisetin is the ligand that provides best structural stability, and Rutin causes a greater conformational variation, but with more hydrogen bond interactions. A detailed absorption, distribution, metabolism, and excretion (ADMET) analysis revealed that FXIIa-IN-4 has the best ADMET profile, while Fisetin is the second. Morin and Rutin, on the other hand, were found to have less clear toxic effects. Taken together, although the *in vitro* chromogenic assessment results demonstrated that Rutin has superiority in inhibition of FXIIa enzyme activity, the overall obtained data indicated the importance of Fisetin among all tested flavonoid compounds as an equilibrative natural inhibitor demanding *in vivo* experimental confirmation and focused molecular modification.

Keywords: Fisetin, Morin, Rutin, FXIIa-IN-4, FXIIa, *In Vitro*, *In Silico*

1. Introduction

Blood coagulation is a primitive physiological process to stop the excess blood loss in the case of vessel injury [1]. This intricate process occurs through an enzymatic cascade mediated by plasma coagulation factors and culminates with the establishment of a stable fibrin clot [2]. As this system is necessary for survival, its uncontrolled or wrongful activation may result in severe pathological events such as deep vein thrombosis, ischemic strokes, pulmonary embolisms or myocardial infarctions. These are increasingly prevalent and are indeed major causes of morbidity and mortality worldwide [3].

Among these enzymes, activated Factor XII (FXIIa) occupies a unique position. It is a serine protease which plays a role in the activation of the contact pathway of coagulation, which is activated by a negatively charged surface or by activates such as polyphosphates, nucleic acids or biomaterials [4]. FXII activation to FXIIa results in positive feedback to coagulation through FXI activation, but also plays a role in kallikrein-kinin-system activation and inflammation [5]. In humans, in vivo analyses have demonstrated that, contrary to other enzymes in the cascade, congenital FXII deficiency is not associated with a bleeding diathesis, and this episode supports the idea that FXII is dispensable for normal hemostasis [6].

As this is a non-obligate element of hemostasis and a key player in thrombosis-related disease, FXIIa is an attractive target for the development of novel antithrombotic agents [7]. Blocking the active FXIIa may thus prevent or treat abnormal thrombi without provoking hemorrhages, which is often observed with classical anticoagulants such as heparin or vitamin K antagonists [8]. Therefore, the creation of selective FXIIa inhibitors constitutes a novel strategy, in which potency, safety, and therapeutic selectivity are united. In this light, the identification of new molecules, of either natural or synthetic origin, that can selectively modulate FXIIa activity constitutes a significant biomedical challenge that computational chemistry can now address rationally and predictively [9].

In this context, the research of new FXIIa inhibitors is increasing, with increasing interest for bioactive natural compounds such as flavonoids for their wide pharmacolo-

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gical spectrum, relative bioavailability and low toxicity. The present paper aims to explore the inhibitory properties of a number of compounds, like Fisetin, Morin, Rutin, and a synthetic analog FXIIa-IN-4, employing an in-silico research that integrates several computational chemistry approaches.

Fisetin, Morin, and Rutin are flavonoids known for their potential health benefits, including anti-inflammatory, antioxidant as well and effects on blood coagulation. Moreover, compounds with phenolic structures, like many flavonoids, have been associated with modifying platelet activity and blood viscosity, potentially affecting coagulation times and bleeding risk [10].

Flavonoids such as Fisetin, Morin, and Rutin play a significant role in modulating coagulation processes primarily through their effects on platelet aggregation and fibrin formation. These compounds possess notable antiplatelet activities, which are crucial in preventing abnormal blood clot formation and reducing the risk of thrombotic events. By inhibiting platelet aggregation, flavonoids help maintain vascular integrity and mitigate the likelihood of cardiovascular complications [11]. The mechanism through which flavonoids exert their effects involves influencing vascular function. Specifically, they demonstrate vasodilatory properties that contribute to improved blood flow and endothelial function. This vasodilation is essential for regulating blood pressure and aiding cardiovascular health. The antiplatelet effects of flavonoids further underscore their potential in managing hypertension by promoting vasorelaxation and modulating endothelial function [12]. Overall, while flavonoids like Fisetin, Morin, and Rutin have not been as specifically studied in the context of coagulation, the general understanding of their biological effects suggests they influence clot formation and vascular health through their antioxidant, antiplatelet, and vasodilatory activities [13].

We start by considering the frontier molecular orbitals (HOMO and LUMO) to calculate the energy gap (ΔE) for each compound. This is indicative of electronic reactivity, and then, of the ability to interact with the protein target. Small ΔE is usually responsible for more reactive ones and results in the formation of non-covalent bonds with the active site of the enzyme [14,15].

Moreover, Electrostatic Potential Surface (ESP) maps also make possible the visual inspection of the electronic charge distribution around the ligands, allowing the localization of regions that may electrostatically interact with the protein receptor [16,17,18]. The Non-Covalent Interaction (NCI)analysis further offers visualization of weak interactions (H-bonds, van der Waals forces, π–π stacking, etc.) involved in the stabilization of the ligand–protein complex [19].

Molecular docking is subsequently carried out for the possible spatial orientation of the ligands in the active site of FXIIa and the calculation of binding affinity by means of energy score. This forecast is further improved through an MD simulation, which calculates over time the stability of the complex between the ligand and the protein, considering both thermal fluctuations and conformational contributions in a quasi-realistic surroundings [20].

A parallel analysis is also performed on the Absorption, Distribution, Metabolism, Excretion, Toxicity (ADMET) predictions in order to predict the pharmacokinetic properties and safety of the compound candidates, which are key factors in our preclinical selection process [21]. The multidisciplinary strategy used in the present work attempts, therefore, to identify the appropriate FXIIa inhibitors which would be targeted on the basis of the known electronic, structural, and dynamic, as well as pharmacokinetic parameters to realize in silico rational-aided drug design.

2. Materials and methods

2.1. Materials

Human α-FXIIa was procured from Enzyme Research Laboratories (South Bend, IN, USA). FXIIa substrate, S2302, was obtained from Aniara (Mason, OH, USA). Fisetin, Morin and Rutin were purchased from Sigma-Aldrich (St. Louis, MO, USA). FXIIa-IN-4 was procured from MCE (MedChemExpress, Monmouth Junction, NJ, USA).

2.2. In vitro Chromogenic Assay

The target of this assay was to investigate the potential effects of Fisetin, Morin and Rutin on FXIIa. In conjunction with 0.5 mM S2302, 9 nM of activated FXII was employed. The enzyme FXIIa and the substrate S2302 served as baseline controls for the variable under investigation. The positive control sample comprised of FXIIa and S2302 in a HEPES buffer with a pH of 7.1 with no drug. The negative control consisted of only the HEPES buffer and the appropriate substrate. The experimental concentrations of the drugs utilized in the study were 0.1-100 μM for the reference drug FXIIa-N-4 and 1-1000 μM for the tested compounds (Fisetin, Morin, and Rutin). Following the addition of the HEPES bicarbonate buffer with a pH of 7.1, the total volume of each mixture was adjusted to 330 μL. The substrate was the final component added, ensuring a timely and efficient process. Subsequently, the fluid was pipetted in and out of each tube to ensure thorough mixing. An assay plate was prepared with 100 mL measurements from the sample, which were then distributed into triplicate wells. The plate was incubated at 37 degrees Celsius for 1 hour. The rate of FXIIa inhibition by the drugs was assessed by measuring the change in absorbance at an optical density of 405 nm [22,23]. Data was collected in triplicate from 3 to 4 independent experiments.

2.3. *In Silico* Methodologies

The computing method used in this work combines a range of in silico methodologies to predict inhibition by four ligands, which include three natural flavonoids (Fisetin, Morin, and Rutin) and one synthetic, FXIIa-IN-4, against activated factor XII (FXIIa). The optimized structures generated by the B3LYP/6-31G(d,p) density functions of the GAUSSIAN program were used to calculate the HOMO and LUMO of both ligands to obtain the energy gap (ΔE) as measure of the electronic reactivity [24]. Electrostatic potential surface (ESP) maps were calculated by Multiwfn and constructed by VMD in order to determine the charge distribution and potential location of the sites for electrostatic complementarity with the target protein [25,26]. Non-covalent interaction (NCI) analysis was performed using NCIPLOT to understand the weak intramolecular forces such as hydrogen bonding and van der Waals interaction employing reduced density gradients [27]. Molecular docking Additional simulations were performed using AutoDock Vina, on the FXIIa (PDB ID: 6L63) as

receptor [28]. Protein and ligand preparations involved elimination of solvent molecules, adding polar hydrogens, and charge assignment, and docking results were analyzed based on binding energy scores and visual inspection of important interactions using PyMOL (PyMOL Molecular Graphics System, version 1.5.0.5) and BIOVIA Discovery Studio version 4.5 (BIOVIA LLC) [29,30].

MD simulations were performed in GROMACS 2023.1 using the CHARMM36 force field to validate docking results and evaluate complex stability. Simulations were performed on neutralized and minimized systems solvated by SPC water at 300 K, 100 ns in SPC water under NVT/NPT ensembles, where stability benchmarks such as the RMSD, RMSF, radius of gyration and H-bonding profiles were compound-selected [31,32]. Last, ADMET (absorption, distribution, metabolism, excretion and toxicity) analysis was also performed, including oral absorptions, CYP450 interactions, solubility, hERG inhibition, mutagenicity, and ecotoxicity, allowing a multi-dimensional assessment of the ligands as selective FXIIa inhibitors [33].

2.4. Statistical Analysis

Data were obtained in triplicate from four distinct experimental assays. The mean ± SEMs from the FXIIa chromogenic assays were assessed using GraphPad Prism 10.0 (GraphPad Software, Inc., San Diego, CA, USA) through nonlinear regression analysis. A one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test was utilized to analyze the data. Statistical significance for all comparisons was defined as $p < 0.05$.

3. Results

3.1. Compounds Inhibition of FXIIa Activity

The inhibitory activity of Fisetin, Morin, and Rutin against FXIIa was determined by chromogenic assay. Four separate experiments in triplicates were performed at 1–1000 µM concentrations, using FXIIa-IN-4 as a reference inhibitor. These three flavonoids suppressed FXIIa activity in a dose-response pattern. At 1000 µM, the percentages of inhibition were at 52.6 % (Fisetin), 57.1 % (Morin) and 71.9 % (Rutin). The IC_{50} values for these compounds were 350.47 µM, 353.77 µM and 326.81 µM (Fig. 1A–B).

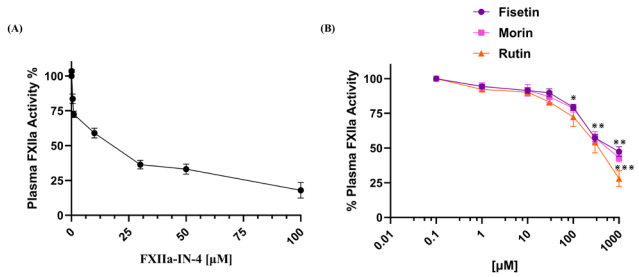

Fig. 1. Effects of flavonoid compounds on human blood coagulation activated factor XII (FXIIa). **(A)** Evaluation of FXIIa-IN-4 on FXIIa activity. FXIIa was incubated with or without increasing concentrations of FXIIa-IN-4 (1–100 µM). **(B)** Examination of the effects of Fisetin, Morin, and Rutin on FXIIa activity. These compounds were tested at various concentrations (1–1000 µM) in the presence of S2302 (0.5 mM). The activity of FXIIa was determined by measuring the rate of S2302 hydrolysis. Results for all panels are expressed as % mean ± S.E.M. from three independent experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ compared to FXIIa-S2302 samples without inhibitor.

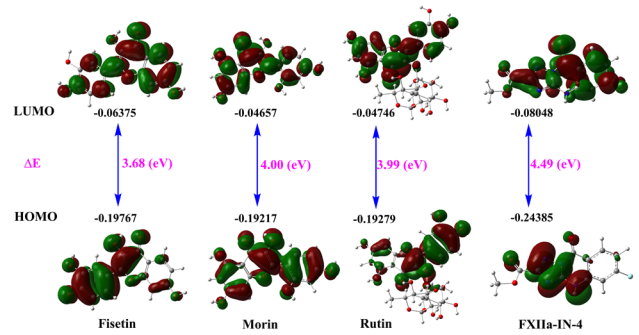

Fig. 2. Distribution of HOMO–LUMO Electronic Densities and Energy Gaps of Studied Ligands.

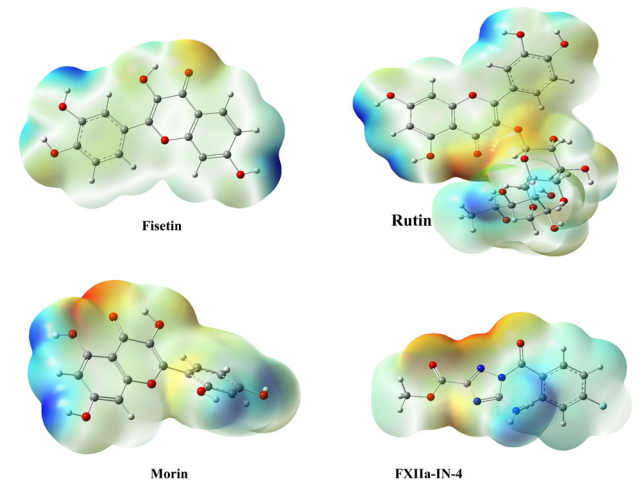

Fig. 3. Electrostatic Potential Maps of the Compounds Studied for Their Affinity towards FXIIa.

3.2. Analysis of electronic structure Using Frontier Molecular Orbitals (FMO)

HOMO–LUMO based frontier molecular orbital (FMO) calculations were also investigated for electronic reactivity. The energy gaps (ΔE) were 3.68 eV for Fisetin, 4.00 eV for Morin, 3.99 eV for Rutin and 4.49 eV for FXIIa-IN-4 (Fig. 2). Smaller ΔE refers to higher electronic reactivity, suggesting that Fisetin could have the maximum interaction preference towards protein target.

3.3. Electrostatic Potential (ESP) Mapping

An electrostatic potential surface has been created to visualize charge distribution. Red indicates electron-rich (negative) sites, and blue represents electron-poor (positive) regions; white/green corresponds to neutral regions (Fig. 3). Fisetin and Morin revealed small polarised masses with negative potentials, localised at the carbonyl and phenole oxygen atoms. Rutin surface was not homogeneous, containing many polar sites, and FXIIa-IN-4 had balanced charge distribution suitable for complementarity in terms of electrostatic.

3.4. Non-Covalent Interaction (NCI) Analysis

Among these interactions, the H–bonds, π–π stacking and van de Waals contacts were revealed by NCI plots (Fig. 4). Several green zones in fisetin indicated that it had well-orientated position and intramolecular hydrogen

bonds. Morin had the same but slightly more scattered interactions. Rutin showed a broad region of green and red, indicative of strong binding, but steric hindrance caused by large glycosidic moiety. FXIIa-IN-4 featured small, tight binding sites with low steric hindrance.

3.5. Molecular Dynamics (MD) Simulations

3.5.1. Root Mean Square Deviation (RMSD)

RMSD profiles were derived from the 100 ns MD simulations (Fig 5). Fisetin showed the lowest range of mean RMSD values (0.05–0.15 nm), suggesting that is a stable complex as compared to other ligands. Moderate difference in Morin and FXIIa-IN-4 (0.10–0.20 nm); Rutin exhibited greater fluctuations (0.20–0.45 nm) , indicating flexibility in conformation.

3.5.2. Root Mean Square Fluctuation (RMSF)

Intramolecular fluctuations were less than 0.3 nm for all complexes except Rutin, which exhibited a maximum around residue 1800 (≈ 0.6 nm) (Fig. 6), revealing increased local flexibility. Fisetin, Morin and FXIIa-IN-4 showed normal profile (with slight perturbations).

3.5.3. Radius of Gyration (Rg)

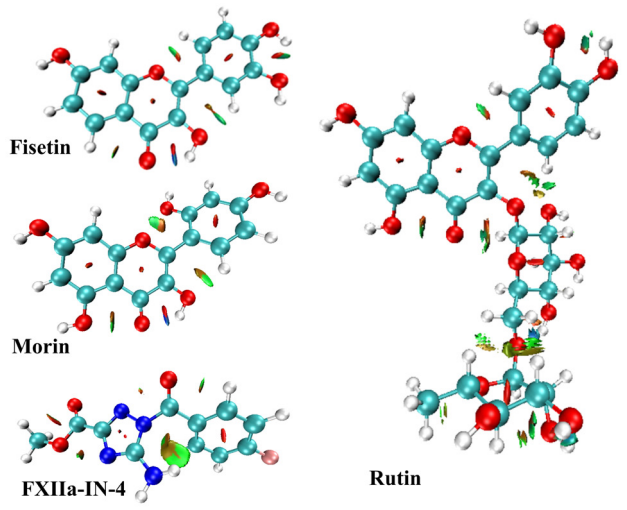

Fig. 4. Analysis of Non-Covalent Forces in Fisetin, Morin, Rutin, and FXIIa-IN-4.

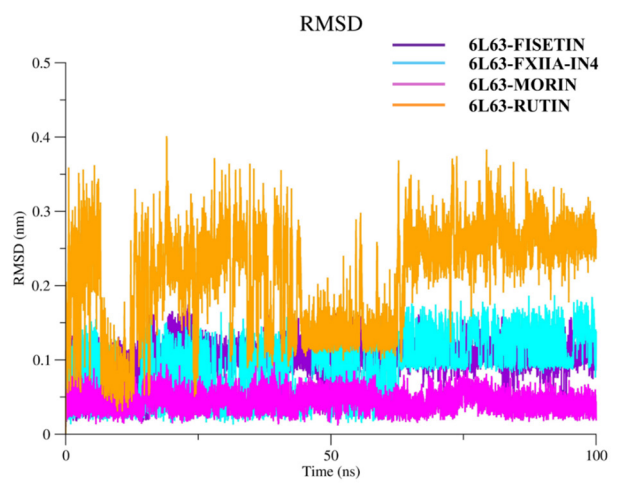

Fig. 5. Structural stability (RMSD) of FXIIa in complex with Fisetin, Morin, Rutin, and FXIIa-IN-4.

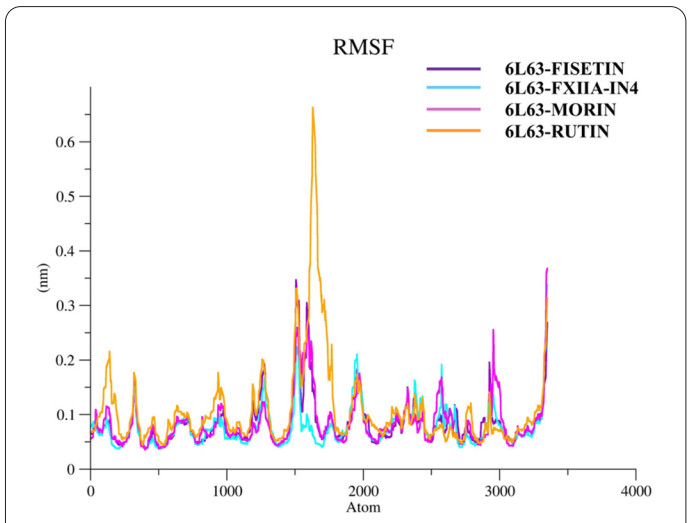

Fig. 6. Residue-level flexibility (RMSF) of FXIIa bound to Fisetin, Morin, Rutin, and FXIIa-IN-4.

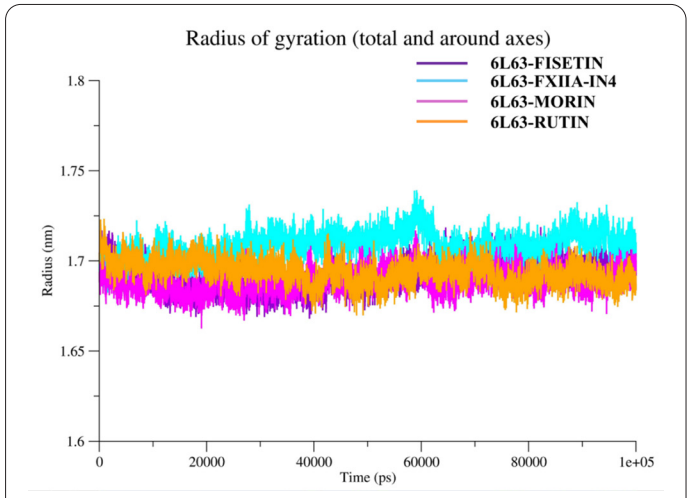

Fig. 7. Radius of gyration profiles of FXIIa–ligand complexes during molecular dynamics simulation.

The Rg values (Fig. 7) show higher compactness in FXIIa–Fisetin (1.68 nm) and FXIIa–Morin (1.70 nm). Rutin had a slightly looser structure (1.70–1.73 nm), while FXIIa-IN-4 displayed intermediate dynamics.

3.5.4. Solvent-Accessible Surface Area (SASA)

SASA plots (Fig. 8) indicated reduced solvent accessibility to FXIIa–Fisetin and FXIIa–Morin complexes (106–112 nm²), while that of FXIIa–Rutin was estimated to be higher (113-125nm²).

3.5.5. Hydrogen Bond Analysis

The amount of hydrogen bonds (Fig. 9) from 1–2 for Fisetin and Morin, 2–3 for FXIIa-IN-4 and 2 to 5 Ferm Rutin with the denser but more heterogeneous network.

3.6. Molecular Docking Analysis

Docking was studied on FXIIa (PDB ID: 6L63). The binding affinities (Table 1) were −8.6 kcaVmol (Rutin), −7.0 kcal/mol (Fisetin), −6.8 kcal/mol (Morin), and −6.4 kcal/mol (FXIIa-IN-4). 2D and 3D visualizations (Fig. 10) showed hydrogen bond with ASP602, GLN581 and GLY382 for Fisetin; TYR481 and LEU527 for Morin as well as several polar residues for Rutin whereas FXIIa-IN-4 established focused interactions with ASP602,

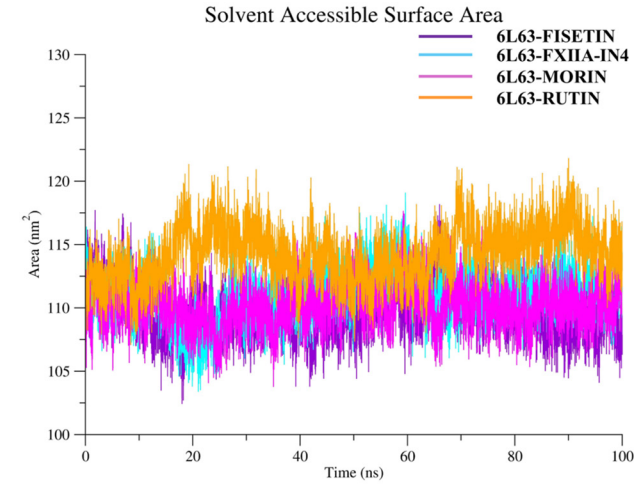

Fig. 8. Solvent-accessible surface area (SASA) profiles of FXIIa–ligand complexes reveal ligand-induced conformational exposure.

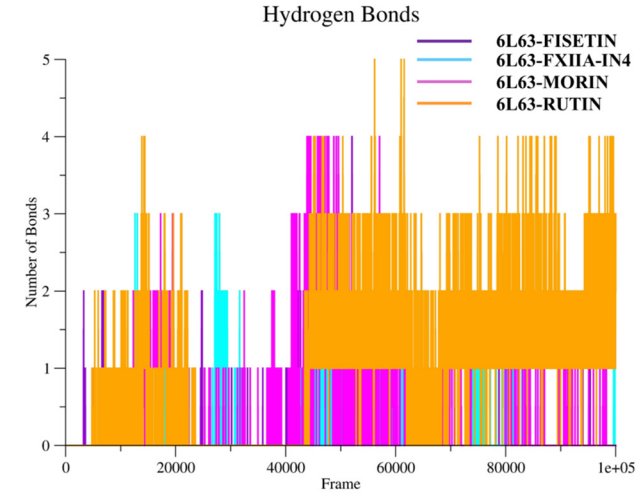

Fig. 9. Hydrogen bonding dynamics of FXIIa–ligand complexes during molecular simulation.

Table 1. Results of the molecular docking of candidate ligands with the FXIIa protein (PDB: 6l63): affinity values in kcal/mol.

Ligands	Affinity (kcal/mol)
Fisetin	-7.0
Morin	-6.8
Rutin	-8.6
FXIIa-IN-4	-6.4

PHE526 and LEU547.

3.7. ADMET and Toxicity Predictions

Pharmacokinetic properties (Table 2) High HIA in human intestinal mucosa were found for FXIIa-IN-4 (88.8 %) and Fisetin (80.8), moderate for Morin (66.8) and poor for Rutin (3.2). BBB permeability was highest for Fisetin (0.57). FXIIa-IN-4 revealed good solubility and a weak CYP450 inhibition.

Toxicity predictions (Table 3) indicated mutagenicity for all the compounds when considering Ames tests. FXIIa-IN-4 had a couple of other positive sub-tests and minimal carcinogenicity in rats. Rutin showed high water toxicity, while Fisetin and Morin had the lowest general toxicity. Pharmacokinetic radar and BOILED-Egg figures (Fig. 11), to validate oral absorption for Fisetin and FXIIa-IN-4.

4. Discussion

4.1. Combined analysis of in vitro and in silico data

Together, the in vitro chromogenic assays and in silico modeling provided complementary information on inhibitory capacity of Fisetin, Morin, Rutin and FXIIa-IN-4 toward human Factor XIIa [34]. The results of the laboratory experiments firmly demonstrated that all of these natural flavonoids are anti-FXIIa in a concentration-dependent manner and Rutin exerted approximately 72% apparent inhibition at 1000 µM. The strong binding can be attributed to its high density of hydroxyl and glycosidic groups, which are favorable for making multiple hydrogen bonds with polar residues in the active site [35]. Nonetheless, in silico studies indicated that such structural diversity also brings steric hindrance and conformational freedom to destabilize the complexation as well as deep-pocket binding. Hence, the good inhibition of Rutin in vitro could be due to some surface interaction or allosteric binding rather than ideal occupancy of catalytic-site. On the other hand, Fisetin was second best in terms of scoring function but was the most stable and energetically favorable ligand from the computational analysis. Its planar and small molecular size, low HOMO–LUMO gap (3.68 eV), and local negative potential, facilitate efficient π-π stacking and hydrogen-bonding interactions with catalytic residues such as ASP602, GLN581, GLY382 [36].

MD trajectories showed slight variation and stable RMSD within 100 ns, revealing that Fisetin exhibits structural stability upon binding to FXIIa. This dual evidence

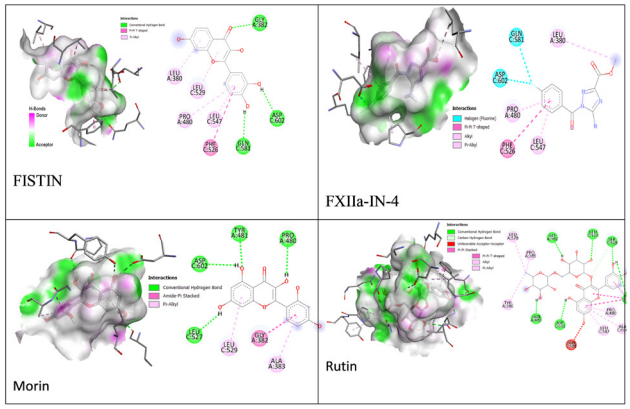

Fig. 10. Visualization of molecular interactions (2D and 3D) between candidate ligands and the FXIIa protein.

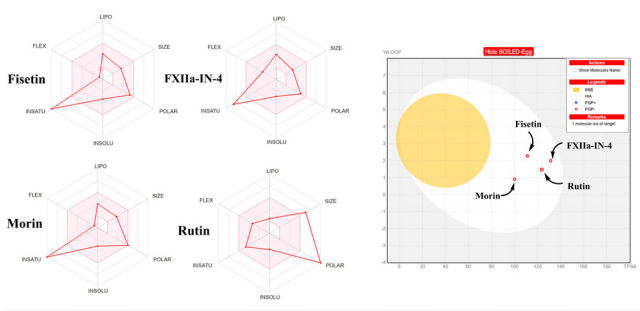

Fig. 11. Predicted pharmacokinetic profiles of FXIIa ligands: SwissADME and BOILED-Egg analyses.

Table 2. In silico evaluation of the pharmacokinetic parameters (ADME) of Fisetin, Morin, Rutin, and FXIIa-IN-4.

ID	Fisitin	Morin	Rutin	FXII-IN-4
BBB	0.566596	0.286931	0.0296906	0.282038
Buffer_solubility_mg_L	139,748	77,0733	112,347	487699
Caco2	18,49	20,289	8,77131	14,217
CYP_2C19_inhibition	Inhibitor	Inhibitor	Inhibitor	Non
CYP_2C9_inhibition	Inhibitor	Inhibitor	Inhibitor	Non
CYP_2D6_inhibition	Non	Non	Non	Inhibitor
CYP_2D6_substrate	Non	Non	Non	Substrate
CYP_3A4_inhibition	Inhibitor	Inhibitor	Inhibitor	Non
CYP_3A4_substrate	Weakly	Weakly	Weakly	Weakly
HIA	80,773991	66,789451	3,159758	88,763063
MDCK	247,028	31,202	0.0517434	114,026
Pgp_inhibition	Non	Non	Non	Non
Plasma_Protein_Binding	94,038813	97,582151	61,702822	38,376727
Pure_water_solubility_mg_L	365,609	1553,58	443,354	1741,05
Skin_Permeability	-4,12767	-4,25107	-4,47234	-4,05656
SKlogD_value	2,06961	1,54249	-0,66458	-1,69149
SKlogP_value	2,06961	1,54249	-0,66458	0.136640
SKlogS_buffer	-3,31145	-3,59351	-3,72508	0.267820
SKlogS_pure	-2,89378	-2,28908	-3,12889	-2,17952

Table 3. Predictive evaluation of the toxicity of candidate ligands for FXIIa inhibition.

ID	Fisitin	Morin	Rutin	FXII-IN-4
algae_at	0.0399242	0.0303054	0.00389677	0.172102
Ames_test	mutagen	mutagen	mutagen	mutagen
Carcino_Mouse	negative	negative	negative	negative
Carcino_Rat	negative	positive	negative	positive
daphnia_at	0.173085	0.17818	1,1518	0.452111
hERG_inhibition	medium_risk	medium_risk	ambiguous	medium_risk
medaka_at	0.0481279	0.052536	2,54783	0.29213
minnow_at	0.0313091	0.0323288	1,73292	0.241811
TA100_10RLI	negative	negative	negative	positive
TA100_NA	negative	negative	negative	positive
TA1535_10RLI	negative	negative	negative	positive
TA1535_NA	negative	negative	negative	positive

indicates that Fisetin is involved in a strong, specific interaction that could lead to long-lasting inhibitory activity under physiological conditions. Morin, sharing a similar flavonol backbone, showed intermediate performance. Its energy gap (4.00 eV) and ESP map point to slightly weaker electron delocalization, explaining its modest binding affinity (−6.8 kcal/mol) and moderate conformational stability in MD simulations. Nonetheless, Morin still produced a stable complex, reinforcing that small variations in hydroxyl orientation can significantly influence recognition and stabilization of FXIIa.

The synthetic analogue FXIIa-IN-4, as a reference compound, showed different behavior: while extremely soluble in aqueous medium and showing good ADMET scores, it had the lowest docking affinity (−6.4 kcal/mol), and an intermediate flexibility along MD simulations. This discrepancy indicates that purely physicochemical optimization in rational drug design cannot ensure optimal target complementarity. Further structure-based optimization would be needed, in particular for its aromatic and polar regions to increase anchoring efficiency.

4.2. Molecular basis of binding stability and selectivity

The multilevel computational analysis (FMO, ESP, NCI, MD) was also instrumental in providing further insights into molecular determinants of the FXIIa–ligand recognition [37]. Low ΔE and uniform charge distribution of Fisetin provide for the balance between electronic reactivity and structural compactness that is needed to sustain strong electrostatic and π–π interactions with steric non-overlap. The ESP and NCI analyses suggested further that the carbonyl and hydroxyl groups of Fisetin are both hydrogen-bond donors/acceptors, which serve as stable anchoring points inside the catalytic cleft. On the other hand, Rutin's bulky disaccharide substituent increased polarity as well as steric bulk and provoked repulsive interactions, illustrated by red regions in the NCI plots. This accounts for its RMSD and Rg being high, and overdue to also producing the local unfolding of the protein (observed from SASA change). Therefore, Rutin can form

more transient hydrogen bonds, but the total interaction is dynamic and less efficacious for long-term inhibition. FXIIa-IN-4 had low polar contacts with excellent shape complementarity, suggesting that its binding is driven by van der Waals and hydrophobic stabilization rather than strong electrostatic anchoring. Taken together, these results emphasize that efficient FXIIa inhibition depends on an appropriate balance of molecular reactivity, size and conformational or rigidity.

4.3. Correlation between docking affinity and dynamic stability

Comparison between docking scores and MD parameters sheds light on an important point; a high affinity of the docked pose is not necessarily indicative of dynamic stability [38]. Although Rutin showed best docking energy (−8.6 kcal/mol), it also had highest RMSD, RMSF and SASA values which was indicative of the worst conformational retention; Fisetin had superior compactness (Rg = 1.68 nm) and least solvent accessibility but with a weak binding affinity (−7.0 kcal/mol), indicating that the complex is folded more tightly over the time frame. These results show that energy alone is not sufficient to predict inhibition strength and requires the support of dynamic stability measures. This kind of docking-MD combination thus contributes to the improvement in predictive reliability for identify FXIIa inhibitors.

4.4. Pharmacokinetic and toxicological implications

The ADMET assessment adds a valuable pharmacological perspective to these structure-based observations [39]. Of the compounds tested, Fisetin possessed favorable intestinal absorption (Human Intestinal Absorption (HIA)= 80.8%), moderate BBB permeability and low ecotoxicity with tolerable plasma protein binding (94%). Morin exhibited the same trend but with slightly lower absorption. Rutin's low permeability and low HIA (3.2%) eliminate it from oral application; FXIIa-IN-4 showed in predictive toxicity screens some potential genotoxic and carcinogenic effects despite excellent solubility and only very little CYP inhibition. These observations highlight that Fisetin has the highest overall balance between pharmacokinetics and the toxicological profile, consistent with its structural and energetic stability. The moderate lipophilicity also ensures good bioavailability, avoiding excessive accumulation in lipid stores.

4.5. Mechanistic insight and therapeutic perspectives

Mechanistically, Fisetin and Morin would inhibit FXIIa by the interaction in a competitive manner with its catalytic domain, putting both π–π stacking and hydrogen bonding in play to a similar extent. Rutin's elongated structure may suit peripheral or allosteric binding with reversible inhibition [40]. Combining the quantum chemical descriptors (ΔE, ESP) together with the molecular docking and dynamic behavior of compounds is evidence that all these multidimensional computational approaches could be used as a preclinical tool for screening coagulation inhibitors. The consistent results at the electronic, structural and pharmacokinetic scales bear witness to the robustness of the strategy, while offering mechanistic explanations why Fisetin was chosen as a lead scaffold for subsequent optimization. Minor chemical modifications, such as specific hydroxyl methylation and halide substitution in substrate analogues, might be expected to increase its potency and selectivity toward FXIIa while preserving low toxicity.

4.6. Study limitations and future directions

Despite the predictive power of the integrated in vitro–in silico approach, however, there are limitations to consider. No in vivo validation of either anticoagulant or anti-inflammatory efficacy was performed, which is essential to validate the physiologic relevance of FXIIa inhibition [41]. In addition, modeled solvation environments may not be as realistic as the true dynamic conditions for plasma enzymes and the models assume a fixed conformation for the receptor in vacuo. Future studies should focus on: performing ex vivo plasma clotting and thrombin-generation assays to verify the inhibition pathways; studying SAR among flavonoid derivatives, and chemically modifying Fisetin analogs by molecular hybridization design or pharmacophore screening; testing toxicity in animal models along with pharmacokinetics of newly predicted compounds.

5. Conclusions

The overall analysis conducted in this study demonstrates the relevance of an in vitro as well as in silico approach combining quantum chemistry tools, structural modeling, and ADMET prediction to identify potential inhibitors of the FXIIa protein. The results allowed for the ranking of the tested compounds according to several criteria: electronic reactivity, electrostatic complementarity, conformational stability, binding affinity, pharmacokinetic properties, and predicted toxicity. Through the data obtained from the in vitro chromogenic investigations, Rutin exhibited the strongest inhibitory effect, while Fisetin and Morin showed almost similar inhibitory percentages on the activity of FXIIa. From in silico studies, Fisetin emerges as the most promising natural candidate due to its balanced overall profile, combining good affinity, structural accessibility, and relative toxicological safety. FXIIa-IN-4, a synthetic molecule designed to specifically target FXIIa, exhibits excellent solubility and strong predicted absorption, but requires structural adjustments to improve its affinity and mitigate its mutagenic risks. On the other hand, Rutin, although displaying remarkable affinity, is penalized by its molecular bulk and poor absorption, while Morin remains an intermediate candidate. The overall results indicate that Fisetin suggests that it is the most suitable natural flavonoid candidate that could be utilized as a lead compound for developing more potent therapeutic agents for the purposes of management or prevention of FXIIa-associated thrombo-inflammatory diseases. These results also lay the groundwork for future in vivo experimental studies and pave the way for the rational design of new anticoagulant inhibitors targeting FXIIa, at the intersection of bioinformatics, pharmacochemistry, and structural biology.

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Conflict of interest
No conflict of interest.

Data availability
All data are included in the manuscript.

Ethics approval and consent to participate

Not Applicable.

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Authors' contributions

HAM designed the research study. HAM performed the research. HAM analyzed the data. HAM wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors have read and approved the final manuscript.

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