Review:

A Deep Overview of Anticoagulant Drugs: Recent Synthesis and Their Activity Assay

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Abstract: *During the unprecedented COVID-19 pandemic, anticoagulant drugs have emerged as a crucial component of treatment alongside antivirus medications. Patients with severe COVID-19 frequently have critical conditions marked by blood clot development, necessitating the administration of anticoagulants. This review aims to provide a comprehensive overview of various anticoagulant drugs, their synthesis methods, and assays employed to predict their anticoagulant activity. Notable anticoagulant categories frequently utilized include oral anticoagulants heparin, non-vitamin K antagonists, and vitamin K antagonists. In recent years, the development of new anticoagulants has seen a shift towards a multifaceted approach that combines* in silico *prediction with* in vitro *and* in vivo *assays.* In silico *prediction techniques play a pivotal role in the initial screening process. This integrated approach has yielded promising results, paving the way for the synthesis of novel anticoagulant candidates, as substantiated by a battery of* in vitro, in vivo, *and* ex-vivo *tests.*

Keywords: *anticoagulant; prediction; synthesis; anticoagulant activity*

■ INTRODUCTION

Anticoagulants are drugs that are used to prevent some factors in the coagulation cascade. This drug is used in clinics to prevent and treat ischemic stroke, venous thromboembolism (VTE), pulmonary embolism (PE), and deep vein thrombosis (DVT), where these three conditions together account for the majority of cardiovascular deaths [1-2]. Recently, it has been widely used for COVID-19 patients with blood clot incidents. Several oral anticoagulants are often used for VTE by inhibiting thrombin, inhibiting factor Xa and other mechanisms [3].

Anticoagulant therapy needs continuous monitoring to avoid side effects of bleeding and allergies. Heparin has several disadvantages, including the danger of pathogens contamination, bleeding, thrombocytopenia, bruise, contact dermatitis, hives, and epithelium necrosis [4]. Anticoagulant drug usage is increasing due to cardiovascular problems in the community and an increasingly elderly population has the highest yearly growth rate among the top ten care areas. Therefore, prompted researchers are seeking and developing new and improved anticoagulants [5].

Several researchers discovered new anticoagulant

drugs using synthesis by modifying with certain grup added or from natural materials [6]. The new drugs were tested for anticoagulant activity through *in vivo*, *in vitro*, or *in silico* assays [7-14]. *In silico* is known to be able to reduce failures in laboratory experiments by screening potential candidates using computational design for further synthesis. Another advantage of *in silico* is that it can fill data gaps in chemical risk assessments [15]. Some *in silico* models today are molecular docking and molecular dynamics simulations [16-17]. Some of the software used for molecular docking will be shown in this review, along with the PDB code for the anticoagulant protein used. This review will display the synthesis method and all anticoagulant assays through *in vitro*, *in vivo*, and *ex vivo*. This review aims to provide a comprehensive overview of the various types of anticoagulant drugs, their synthesis methods, and the tests used to predict anticoagulant activity so that it can help researchers who will develop new anticoagulant drugs.

■ METHOD

We used renowned Scopus databases (http://www.scopus.com/). The keywords used were "synthesis of anticoagulant" and "activity assay of anticoagulant" within 2018–2022, document-type articles and source-type English journals. VOSviewer was used for the analysis keyword after sorting manually (Fig. 1).

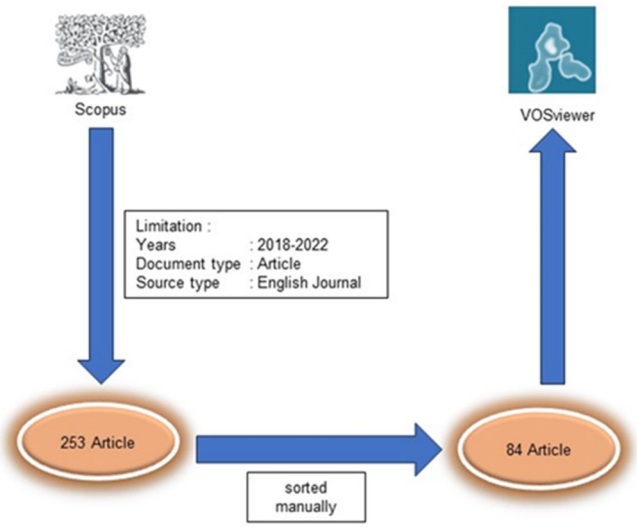

Fig 1. Method step

■ RESULTS AND DISCUSSION

Anticoagulants

Anticoagulants are drugs proposed for critically ill conditions with the exact pathophysiology of hypercoagulability and inflammatory diseases such as acute respiratory distress syndrome (ARDS) and sepsis. An observational study conducted by Ceccato et al. [18] showed that anticoagulants or high prophylactic doses of heparin have been linked to enhance patient outcomes with non-critical diseases. Anticoagulation is not related to improved outcomes in critically sick patients, and alternative treatments such as antiplatelet therapy, fibrinolytic therapy, or nebulized anticoagulants are necessary [18]. The traditional use of anticoagulation has been carried out by doctors in collaboration with laboratories inside or outside the hospital. On self-administered anticoagulants, the patient performs his international normalized ratio (INR) test with a treatment device and only consults a physician for interpretation and dose adjustment [19]. Anticoagulant medication is a critical component that has a significant influence on the thromboembolic possibility of patients with nonvalvular atrial fibrillation (NVAF).

The Global Anticoagulant Registry in the Field-Atrial Fibrillation (GARFIELD-AF) did a profile analysis of subjects with NVAF taking antithrombotic medication orally. Observations resulted in adherence strategies correlated with stroke. Patient noncompliance with oral anticoagulants is the main side effect of ineffective therapy. Toma et al. [20] researched mobile phone applications focused on patient needs and telemedicine applications that track patient progress and identify adverse responses or events that significantly influence treatment adherence.

Oral anticoagulants are effective and safe for antithrombotic prophylaxis following major orthopedic surgery. Somehow, there is little data on their use in cancer surgery. Randomized trials and many meta-analyses in cancer patients receiving open abdominal or pelvic surgery have shown that antithrombotic therapy should be continued for four weeks following surgery to lower the risk of venographic DVT and pulmonary

embolism after one week of administration. According to this information, international guidelines suggest four weeks of low molecular weight heparin (LMWH) prophylaxis following open surgery for abdominal or pelvic cancer surgery [21].

The coagulation system is divided into two routes: extrinsic and intrinsic. Tissue factor (TF) stimulates the extrinsic pathway in the vascular. Coagulation factor XII (FXII) is activated through the intrinsic route. The extrinsic or intrinsic pathway will then initiate downstream coagulation factors until prothrombin is converted to thrombin, resulting in fibrin [22]. Some anticoagulant treatment alternatives, such as unfractionated heparin (UFH) and LMWH, impede coagulation by increasing antithrombin's neutralizing effect on thrombin and FXa. Vitamin K antagonists with a mechanism of action inhibiting the liver's synthesis of coagulation factors II, VII, IX, and X and oral anticoagulants are non-vitamin K antagonists (NVKA) that inhibit thrombin or FXa directly [23].

Types of Anticoagulants

Oral anticoagulant non-vitamin K antagonist

Dabigatran, rivaroxaban, apixaban, betrixaban, and edoxaban are oral anticoagulant non-vitamin K that act as thrombin inhibitors. The cytochrome-P450 (CYP) enzyme, namely CYP3A4, which interacts with P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), is responsible for drug metabolism. Co-administration of medications with these three proteins' substrates, inducers, activators, or inhibitors can influence plasma concentrations, efficacy, and safety [24].

Dabigatran is an anticoagulant that inhibits thrombin orally, thereby blocking fibrin formation. Like drugs of this class, dabigatran also causes platelet aggregation inhibition and reduces the activity of factors V, VIII, and XI. They were usually used for NVAT patients to lower the risk of stroke. It is also utilized in therapy and secondary VTE and prophylaxis of VTE after total hip arthroplasty [25]. The use of dabigatran carries a high risk with little benefit in individuals with mechanical prosthetic valves. In an observational study from Cho et al. [26] on patients with mitral stenosis (MS) and atrial fibrillation (AF), it is recommended to use dabigatran, although no prospective data are available. Dabigatran has the chemical name of *N*-[[2-[[[4-(aminoiminomethyl) phenyl] amino] methyl]-1-methly-1*H*-benzymidazole-5-yl] carbonyl]-*N*-2-pyridinyl β-alanine. Dabigatran has poor oral bioavailability (6–7%) due to its high polarity, so its prodrug is widely used, namely dabigatran etexilate, which is easily absorbed in the digestive system [27]. The log P of dabigatran is 2.17, while dabigatran etexilate has a log P of 5.17. Dabigatran is very soluble in water.

Rivaroxaban is an anticoagulant that inhibits the small molecule FXa, a protease required in the cascade of coagulation and activator of protease-activated receptor 2 (PAR2). One study showed that rivaroxaban prevents atherosclerosis by inhibiting FXa-PAR2-dependent autophagy. Coagulation proteases may promote atherosclerosis by activating PAR2 [28]. Rivaroxaban is the only substance authorized to reduce the number of cardiovascular incidents in subjects with peripheral arterial and coronary artery disease [25]. Rivaroxaban is widely used in several countries because of its advantages as an oral treatment that does not require active surveillance. Rivaroxaban has no adequate and widespread reversal medication if significant bleeding occurs. The efficacy of medicines to prevent VTE must be balanced against possible consequences such as substantial bleeding. Rivaroxaban was more effective than enoxaparin in avoiding DVT and significant VTE in patient undergoing complete joint arthroplasty without worsening bleeding or all-cause mortality in patients undergoing total joint arthroplasty. Rivaroxaban has good oral bioavailability and predictable pharmacokinetics, making it a safe drug for general usage [29]. Rivaroxaban does not cause more bleeding during comprehensive prevention of venous thromboembolism in colorectal cancer patients after laparoscopic surgery [21].

Apixaban is an anticoagulant drug with a low threat of major bleeding, such as edoxaban and dabigatran. Edoxaban carries a decreased risk of systemic embolism and ischemic stroke in atrial fibrillation subjects than oral NVKA and vitamin K antagonists (VKA). Edoxaban also has a reduced chance

of major gastrointestinal bleeding compared to rivaroxaban and VKA [30]. Apixaban should be administered with caution in the presence of potent inducers of CYP3A4 and P-gp, such as phenytoin, carbamazepine, phenobarbital, rifampicin, or St. John's wort [31]. Edoxaban has a higher molecular weight than rivaroxaban, apixaban, and betrixaban, so it has a lower bioavailability than the three drugs. Edoxaban is slightly protonated, determining its moderate bioavailability *in vivo* (62%) at physiological pH. CYP enzymes metabolize apixaban and edoxaban but have a limited capacity to inhibit and induce CYP enzymes, so they are not as susceptible to drug interactions as rivaroxaban [32].

Betrixaban has two nearly planar amide groups, so the water solvent has no effect on the overall form of betrixaban. Betrixaban has an ionizable group. Therefore, food can influence its administration, and its identification by HPLC requires adjustment of the mobile phase PH [32]. Betrixaban is the only one authorized by the FDA to prevent venous thromboembolism over the long term in patients with acute medical conditions. However, it has not been approved by the FDA for any additional indications. Betrixaban is administered once daily with renal elimination reduced, reducing venous thromboembolism risk without increasing serious hemorrhage risk. Betrixaban absorbs quickly, with peak plasma concentrations taking place within three to 4 h, but taking betrixaban with food can reduce peak concentrations by 70% [33].

Vitamin K antagonists

Warfarin is an anticoagulant drug with a VKA mechanism of action. Anticoagulants have severe bleeding complications, so vitamin K, fresh frozen plasma or prothrombin complex concentrate can be utilized to treat bleeding [34]. Warfarin has substantial dose-response variability between people and a restricted therapeutic range of 2.0–3.0 for most indications, according to the international normalized ratio (PT-INR). However, warfarin is the world's most widely prescribed anticoagulant drug [31].

Heparin

Heparin is a polydisperse polysaccharide derived from the intestinal mucosa of pigs. Heparin requires the 3-*O*-sulfation structure to generate a particular pentasaccharide domain that binds antithrombin with high affinity, thereby providing anticoagulant activity. The heparin chain consists of 1,4-linked disaccharide repeat units consisting of uronic acid and glucosamine residues that span a wide range of chain lengths [35-39]. The mechanism action of heparin is to bind to antithrombin III (AT), serine protease inhibitors, thrombin and FXa, which changes conformation to strengthen AT's inhibitory activity. The main forms of heparin are intravenous drugs, UFH, MWavg 16,000 Da; several kinds of subcutaneous LMWH, MWavg 3,500–6,000 Da and subcutaneous ultra-LMWH, MWavg < 2,000 Da [40].

Fondaparinux is a synthetic pentasaccharide that inhibits FXa, preventing thrombin production. Fondaparinux has enhanced pharmacokinetic and pharmacodynamic properties, including FXa selectivity and specificity, full subcutaneous absorption, and a long half-life for once-daily treatment. Subcutaneously given LMWH is a functional fragmented heparin made by chemical or enzymatic depolymerization of UFH. However, there are various benefits related to improved bioavailability, consistent anticoagulant action, the convenience of administration, longer half-life, no need for monitoring, fewer heparin-induced thrombocytopenia, and reduced risk of osteoporosis. Dalteparin is one of the LMWH products produced by fragmentation of HONO heparin followed by borohydride reduction, forming a 2,5-anhydro-mannitol ring at the reducing end [35,41]. Shorter chains exhibited a reduced affinity for plasma proteins (except antithrombin), macrophages, platelets, endothelial cells, platelet factor 4 and osteoblasts compared to UFH [42]. Enoxaparin is an LMWH that must be given twice a day and requires an adjustment of the dose for each patient's weight [43]. LMWH during COVID-19 cases is widely used to lessen the occurrence of cytokine storms in COVID-19 patients hospitalized with severe symptoms. LMWH is also used with mechanisms other than anticoagulants, such as anti-inflammatory/immunomodulatory, antiviral, growth factor modulation, and anticancer effects [44-46]. Ultra-

LMWH controls anticoagulant action better than UFH and LWMH and has the lowest bleeding rate. Ultra-LMWH offers several benefits, but its high cost and inability to be eliminated by means other than renal clearance limit its usage in renal failure with impaired or considerably reduced renal clearance [42].

Synthesis of Anticoagulant Drug

Searching for research articles using the keyword "synthesis anticoagulant" in the Scopus database with limitations in 2018–2022, document-type articles and source-type English journals obtained 253 articles and sorted manually, and obtained 84 articles. The article was processed using VOSviewer software. For extracting data, mapping, and grouping collected papers, VOSviewer 18 software (van Eck and Waltman, Leiden University, Leiden, Netherlands) was utilized. Colored circles represent keywords. The circle size depends on the title and abstract keywords, as seen in Fig. 2. This study's keyword analysis is a solid beginning point for evaluating their evolution in this subject. Keywords characterize the author's focus and achievements and provide a general idea of research trends [47].

VOSviewer's co-occurrence network analysis tool assessed research literature keywords on synthesis anticoagulant action. This analysis required six keyword

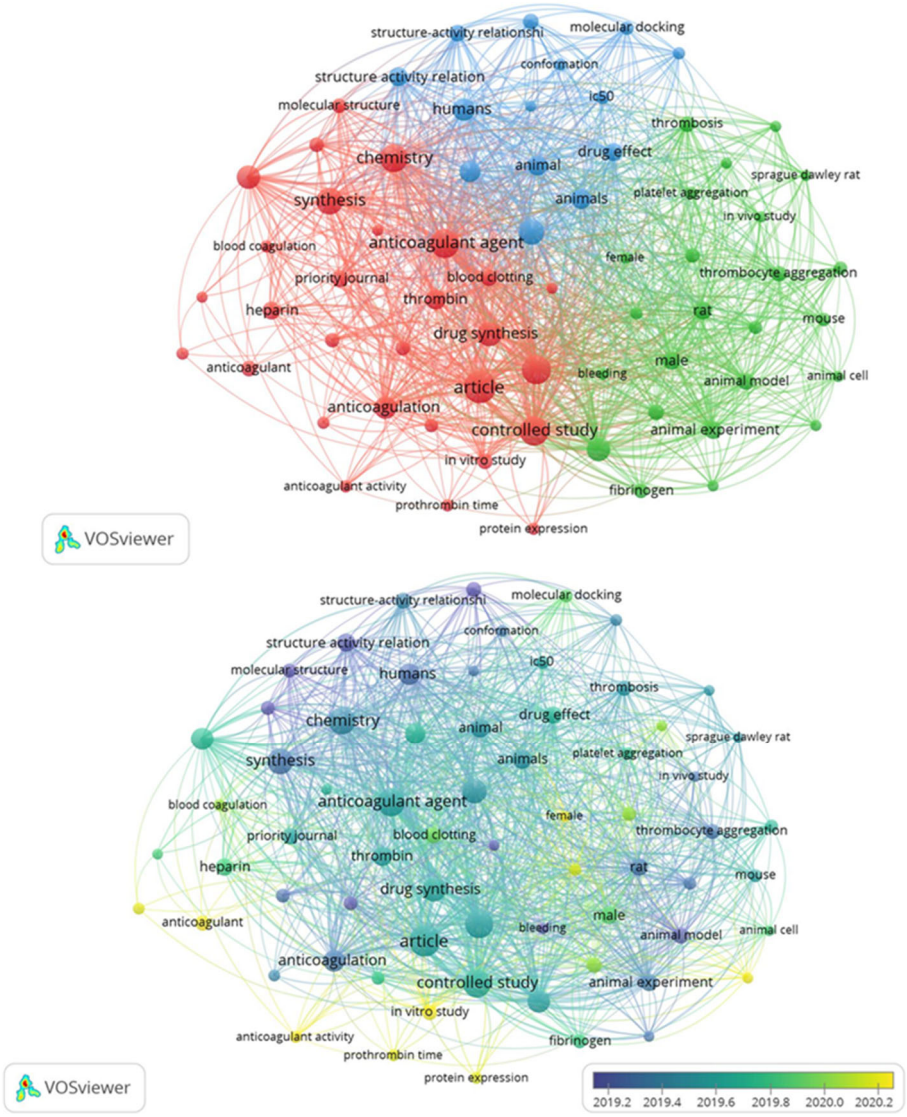

Fig 2. The analysis of keyword co-occurrence on synthesis anticoagulant

appearances in the title and abstract. The system found 67 keywords. The results identified 3 clusters containing the topics shown in Table 1. The most often used keywords in selected papers with the highest link strength were "article" (47 occurrences), "anticoagulant agent" (40), "controlled study" (39), "unclassified drug" (38), and "chemistry" (36).

Several syntheses were carried out to obtain new anticoagulant drugs by researchers such as Hussain et al. [1], where antisense oligonucleotides can prevent thrombotic events in orthopedic surgery patients who are better than enoxaparin in terms of bleeding after being designed to downregulate FXI. In this study, it was devised, synthesized, and evaluated for its ability to inhibit FXIa blood coagulation selectively. The 4,4-disubstituted proline analogs are substituted with 38 groups at various positions. Modeling and designing these structures resulted in a steady increase of FXIa potency by suppressing thrombin activity, and it also increases the selectivity of thrombin [1]. Under homogenous circumstances, Chen et al. [48] employed hemicellulose bagasse to dissolve in alkali disulfation utilizing chlorosulfonic acid and *N*,*N*-dimethylformamide/LiCl. The flow technique obtains a fast, light and efficient xylan sulfate synthesis method. The results revealed that Xylan chain degradation and reaction time were reduced under the flow system, and a high molecular weight product (Mw = 148.217) with a decent degree of substitution (DS = 1.49) was produced at room temperature in 10 min. This study opens up new possibilities for producing alternative functional polysaccharide derivatives that work as anticoagulants under flow reaction circumstances [48].

Mao et al. [49] created one-pot bio-carbon nanowires from natural sodium alginate at low temperatures without a catalyst using *in situ* carbonization and sulfation/sulfonation with solid-state heating. A mixture of sodium alginate and ammonium sulfite with a mass ratio of 5 forms core-shell sulfated/sulfonated bio-carbon nanowires with much stronger anticoagulation activity than sodium alginate and natural sulfated polysaccharides like fucoidan after 3 h of heating at 165 °C [49]. Several synthesis methods, starting compounds and synthetic compounds used to produce new anticoagulant compounds can be seen in Table 2, along with their activities as anticoagulants. Recent research uses green biofunctional production of magnesium oxide (MgO) nanoparticles from *Tarenna asiatica* fruit aqueous extract to synthesize anticoagulants (TAFEMgONPS). TAFEMgONPS increases platelet-rich plasma clotting time, prolongs APTT and PT clot formation, and inhibits ADP-induced platelet aggregation [50]. Nanotechnology employs a wide range of particles that differ in size, shape, and content.

Table 1. Cluster of the research article "Synthesis Anticoagulant" by VOSviewer

Cluster	Total Items	Items
1	29	Activated partial thromboplastin time, anticoagulant, anticoagulant activities, anticoagulant activity, anticoagulant agent, anticoagulants, anticoagulation, antithrombin, article, blood clotting, blood coagulation, chemical structure, chemistry, controlled study, drug screening, drug structure, drug synthesis, heparin, *in vitro* study, molecular structure, nuclear magnetic resonance spectroscopy, polysaccharides, priority journal, protein expression, prothrombin time, proton nuclear magnetic resonance, synthesis, thrombin, unclassified drug
2	24	Animal cell, animal experiment, animal model, antiplatelet activity, antithrombotic agent, antithrombotic activity, bleeding, bleeding time, female, fibrinogen, human cell, *in vivo* study, male, mouse, non-human, platelet aggregation, platelet aggregation inhibitors, rat, rat Sprague-Dawley, signal transduction, Sprague-Dawley rat, thrombocyte activation, thrombocyte aggregation, thrombosis
3	14	Animal, animals, conformation, drug design, drug effect, human, humans, IC_{50}, metabolism, molecular docking, molecular docking simulation, molecular dynamic, structure-activity relation, structure-activity relationship

Table 2. Synthesis of new anticoagulant compound

Initial Compound	Synthetic Compound	Synthesis method	Anticoagulant activity	Test	Type Test	Ref.
2-acetyl pyrazine	2,2'-[4-(*N*,*N*',*N*"-trimethoxyphenyl)pyridine-2,6-diyl]dipyrazines	facile one-pot procedure	all substances prolonged citrated human plasma coagulation in PRP and PPP	*In vitro*	Platelet aggregation, direct haemolytic activity	[52]
2-amino22123-cyanopyrane	pyranopyrimidines 3a-f & pyranotriazolopyrimidines 4a-d	one-pot three-component reaction	all produced chemicals work as anticoagulants	*In vitro*	APTT	[53]
chitosan powder	succinyl- and glutaryl-chitosan derivatives	heterogeneous & homogeneous reactions	exhibit antiplatelet and anticoagulant activity	*In vitro*	Platelet aggregation, APTT, PT, TT	[54]
chitosan	mono- and disulfonic derivatives of chitosan	reductive amination reaction	some sulfonated chitosan extended better than negative control	*In vitro*	APTT, PT, anti-FXa	[14]
ulvan	ulvan-kappa-carrabiose hybrid polysaccharides	ligation	does not increase anticoagulant properties	*In vitro*	APTT	[13]
fondaparinux	derivatives Rrt1.17	a convergent [3+2] coupling approach, orthogonal protecting groups, and various glycosyl donors	active as an anticoagulant and more efficient than fondaparinux	*In vitro, in vivo*	Anti-FXa Antithrombotic effect test *in vivo*	[12]
Oyster (*Crassostrea gigas*) pepsin hydrolysate	food-derived anticoagulant heptapeptides	alkaline extraction (pH 12-13) and acid precipitation (pH 4.8–5.1) were used to isolate it	an alternative food-derived anticoagulant peptide for thrombosis prevention	*In vitro, in vivo*	TT, Fib, APTT, PT	[55]
Dabigatran	11 novels of dabigatran derivatives (12a–12k)	heating, reflux, chlorination, acylation, hydrolysis	the ten compounds obtained showed comparable activity to dabigatran except 12i	*In silico, in vitro*	IC_{50} inhibition of thrombin activity	[56]
O-*tert*-butyl-L-Serine, L-Glutamic acid 5-benzyl ester and L-Cysteine	series of anionic poly(amino acid) s poly (L-Serine-ran-L-glutamic acid-ran-L-cysteine-SO_3)	the controlled ring opening polymerization	polypeptides offer a lot of potential for long-acting anticoagulation	*In vitro*	APTT, PT, TT, Fib, PRT	[2]
ethyl 1-(4-methoxyphenyl)-7-oxo-6-(4-(2-oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydro-1*H*-pyrazolo[3,4-c] pyridine-3-carboxylate	tetrahydropyrazolopyridone derivatives contain 1,3,4-triazole, triazolylmethyl, and partially saturated heterocyclic moieties as P2 binding element	hydrolysis, reduction, chlorination, condensation, and cyclization reaction	all compounds had moderate to considerable potential anticoagulant action, with compound 15c having a 98% inhibitory rate.	*In silico, in vitro, in vivo*	PT, APTT, anti-FXa, *in vivo* antithrombotic effect and inhibition rate	[7]
Porphyra yezoensis	VITPOR AI, a 16-mer peptide	isolated	the peptide inhibited FXIIa amidolysis	*In silico, in vitro*	APTT, PT, TT	[57]
Chitosan	chitosan derivatives	nucleophilic substitution reaction, electrophilic substitution	the quaternary sulfate/chitosan sulfated derivative is less powerful as an anticoagulant than the *N*-alkyl derivative	*In vivo*	Bleeding time	[5]
warfarin	trimethyltin(IV) and tributyltin(IV) derivatives	interaction of sodium salt and triorganotin chloride	indicate higher DNA binding and fragmentation potential	*In vitro*	Towards DNA binding and fragmentation	[58]
Citrus sinensis mesocarp	chemically sulfated pectin from *Citrus sinensis*	chemical sulfation	effectively anticoagulant	*In silico, in vitro*	Proteomic	[4]
Casein	Novel anticoagulant peptide, AVPYPQR (β-CN, fragment 177-183)	*in vivo* digestion	casein can be a source for preparing bioactive peptides via the gastrointestinal (GI) tract's digestive tract.	*In vitro*	PT, APTT, TT	[59]
Whitmania picture Whitman	thermostable anticoagulant proteins from *W. pigra*	boiling, purification	non-blood-sucking medicinal leeches' anticoagulant efficacy depends on WP-77	*In vitro, in vivo, ex-vivo*	APTT, PT, TT, Fib, PRT, cell viability, Carrageenan-induced chronic thromboembolism	[60]

Initial Compound	Synthetic Compound	Synthesis method	Anticoagulant activity	Test	Type Test	Ref.
dabigatran	fluorinated dabigatran analogs	substituted pyridine rings or substituted phenyl rings	all analogs show effective inhibitory activity against thrombin	*In silico, in vitro, in vivo*	IC_{50} inhibition of thrombin	[8]
Fucus vesiculosus	fucoidan by size and to de- and over-sulfate	fractionated by size using ultrafiltration	higher molecular weight increases procoagulant activity. Below 15 kD, activity is significantly reduced	*In vitro*	APTT, PT, EC 50 thrombin generation (CAT) assays	[61]
betrixaban	anthranilamide derivatives	replace the amidine moiety with piperazinyl and change the carbonyl-amino group sequence in the P1 and P4 motifs	compounds 6y and 7f showed the most FXa inhibitory activity	*In silico In vitro, ex-vivo, in vivo*	FXa and thrombin inhibition, *ex-vivo* PT, APTT, bleeding time	[9]
Chitosan	acylated chitosan sulfate	sulfation	greater anticoagulant activity	*In vitro*	APTT, PT, TT, surface plasma resonance, prothrombinase assay	[62]
green seaweed *Codium vermilara* (Bryopsidales)	a highly sulfated 3-linked -arabinan (Ab1)	isolated	the anticoagulant action of pyranosic sulfated arabinan Ab1	*In silico, In vitro*	PT, APTT, TT	[63]
hydroxy substituted coumarin	new bi-thiacoumarins derivatives	integrated cycloaddition and cycloreversion reactions.	shows anticoagulant activity	*In silico, In vitro*	PRT	[64]
dabigatran	ten new dabigatran derivatives	adding methyl and methoxy groups at various points	compounds 7a, 7d, 7j, and 7k provide activity as anticoagulants	*In silico, In vitro, in vivo*	IC_{50} inhibition of thrombin, an arteriovenous thrombosis	[65]
Bacillus cereus strain AB01	bacifrinase (ΔN24)	error-prone PCR, cloned into pET19b vector, and expressed in E5 coli BL21 DE3 cells	its anticoagulant potency is comparable to that of Nattokinase and warfarin	*In vitro, In vivo*	APTT, PT, Fib, the platelet modulating activity, aggregation of platelets, clot solubilities, BCT	[66]
coumarin derivative E	esculin pentasulfate	sulfation	provides effect as an anticoagulant	*In silico, In vitro, ex-vivo, in vivo*	APTT, PT, TT, Activated clotting time (ACT), clot rate (CR), platelet function (PF) and BCT	[67]
the brown algae *Punctaria plantaginea*	highly sulfated linear fucan derivatives	isolation	in APTT and platelet aggregation tests, prevent clot formation	*In silico, in vitro*	APTT, anti-FXa, platelet aggregation	[68]
dabigatran	fluorinated dabigatran derivatives	inserting a hydrophobic group into the terminal benzene or pyridine ring and adding a fluorine atom to the C-2 position	7c, 7k, 7m, and 7o exhibited comparable inhibitory thrombin activity to dabigatran	*In silico, In vitro, in vivo*	IC_{50} inhibition of thrombin	[69]
pyridine	pyrazole dipyridine analogs	pyridine-coupled pyrazoles	compound 6d inhibits coagulation	*In vitro*	By tube coagulase test	[70]
(2R,4R,5S)- and (2S,4S,5R)-enantiomers of 4-(tert-butyl) 2-methyl 5-(4-bromophenyl)-pyrrolidine-2,4-dicarboxylate	enantiopure *N*-((4-chlorophenyl)thio)acetyl pyrrolidine derivatives	FAM-catalytic methodology	thrombin inhibitor	*in silico*	-	[10]
Casein	casein hydrolysate	*in vitro* simulated GI digestion	strong anticoagulant activity	*In vitro*	TT, APTT	[71]
3-(dimethylamino)-1-(1,2,3,4-tetrahydro-2,2,4,7-tetramethyl-6-quinolinyl)-2-propen-1-one 1	tetrahydroquinoline derivatives	condensation	12 of the 40 micromolar compounds blocking mild FXa have selectivity against trypsin, thrombin, factor IXa, and factor XIa	*In silico, In vitro*	Anti-FXa	[72]

Initial Compound	Synthetic Compound	Synthesis method	Anticoagulant activity	Test	Type Test	Ref.
aptamer	2 'fluoro-RNA aptamers	sequence	powerful anticoagulant by blocking the blood coagulation cascade in its early stages	*In silico*	-	[11]
Radix *Salviae miltiorrhiae* total extracts	cryptotanshinone (Cry), dihydrotanshinone I (Dih-I) and tanshinone IIA (Dih-I)	screening using TAC-HPLC-MS/MS system	has anticoagulant effect	*In silico, in vitro*	APTT, TT, PT	[73]
rivaroxaban	pyrazolyl piperidine analogues 4(a–h)	create several amide compounds that target the piperidine ring	compound 4a demonstrated strong anticoagulant action	*In vitro, in silico*	APTT, PT, anti-FXa	[74]
Tarenna asiatica fruit extract	*Tarenna asiatica* fruit extract MgO nanoparticles (TAFEMgO NPs)	the green biofunctional synthesis	TAFEMgO NPs act as anticoagulants and antiplatelets without toxicity	*In vitro, in vivo*	APTT, PT, TT, inhibit the platelet aggregation, bleeding time, clooting time	[50]
2-hydroxybenzaldehyde and 2-hydroxyacetophenone	new Schiff bases functionalized with amide and phenolic groups	condensation	all the compounds showed procoagulant activity	*In vitro*	Clotting time, platelet aggregation	[75]
Inositol	sulfated chiro-inositol (SCI), a non-saccharide mimetic of heparin	esterification	SCI is a potential FXIa allosteric inhibitor	*Ex vivo, in vivo*	PT, APTT, TEG and hemostasis study (HAS), bleeding tail, arterial and venous thrombosis model studies	[76]
hydrazone	(*E*)-*N*'-(1-(3-oxo-3*H*-benzo[f]chromen-2-yl)ethylidene)benzohydrazide	a Knoevenagel condensation and a ring closure	1c may be used as an anticoagulant to avoid thrombosis	*In vitro, In vivo*	Plasma fibrinogen	[77]
Bacterial cellulose	a microbial nano cellulose-ZnO-Ag (CNCs) composite	nanotechnology	bacterial cellulose gained new activity when it was nanosized and coupled with nanoparticles	*In vivo*	PT, APTT	[51]
CD47	TAX2 peptide	genecust at a purity of 98%	TAX2 as an innovative antithrombotic agent	*in vitro, in vivo*	Platelet aggregation, bleeding times	[78]
Fucoidans	synthetic sulfated a-L-fucoside-pendant glycopolymers	cyanoxyl-mediated free-radical polymerization	activate human platelets	*In vivo*	Platelet aggregation	[79]
chondroitin sulfate	sulfated chondroitin sulfates	synthetic modification of chondroitin sulfate	increasing sulfation levels produced an anticoagulant response	*In vitro*	Clotting time, APTT	[80]
Melaleuca bracteata 'Revolution Gold'	betulinic acid (BA) and 3β-acetoxybetulinic acid (BAA)	isolated, acetylation	BAA has stronger antithrombotic, antiplatelet, and anticoagulant properties than BA	*Ex vivo*	Bleeding tail time, platelet aggregation	[81]
1*H*-indole-3-yl-acetic acid methyl ester	the nano-property of dimethyl 2,2′-[2,2′-(ethane-1,1-diyl) bis(1*H*-indole-3,2-diyl)]-diacetate (DEBIC)	one-pot reaction	inhibiting venous thrombosis and inducing no bleeding side effect	*In vivo*	DVT inhibition, bleeding-reaction	[82]
2-(5-bromo-2,4-dihydro-3-oxo-1,2,4-triazolyl-4)acetic acid	sulfone II	oxidation	the compounds' anticoagulant activity was much lower than that of heparin sodium	*In vitro*	APTT, PT	[83]
isatin	new isatin-3-acylhydrazones	molecular hybridization	anti aggregation activity and high anticoagulant	*In vitro*	APTT, PT, Fib	[84]
Acylated Aminotriazoles	*N*-acylated aminotriazoles	microscale parallel synthetic	*N*-acylated aminotriazoles exhibited anticoagulant properties	*In vitro*	APTT, PT, FXIIa and/or thrombin inhibitors	[85]
carrageenan	carrageenan derivatives containing β-D-GalAp units	oxidized	showed a better anticoagulant effect	*In vitro*	APTT	[86]

Initial Compound	Synthetic Compound	Synthesis method	Anticoagulant activity	Test	Type Test	Ref.
a marine pyran-isoindolone derivative	pyran-isoindolone derivatives F1–F7	chemical alteration of the C-2 and C-20 phenol group moieties, as well as the C-1" carboxyl group	F1-F4 and F6 were shown to have strong fibrinolytic activity	*In vitro*	Anti-thrombotic activity	[87]
tannic acid (TA)	-	-	platelet activity and thrombus development are both inhibited by TA	*In silico, in vitro, in vivo*	APTT, PT, Tail-bleeding time	[88]
curcuminoids	dibenzylidene ketone derivatives	the reaction of cyclopentanone	AK-1a and AK-2a severely prolonged bleeding	*In silico, in vitro, In silico*	Plasma recalcification time, Bleeding time	[89]
poly-amidosaccharide	sulfated poly-amido-saccharides (sulPASs)	anionic ring-opening polymerization (AROP)	increase clotting time by reducing intrinsic coagulation	*In vitro, in vivo, ix vivo*	APTT, PT, FXa inhibition, Bleeding time	[90]
isatin	isatin derivatives	alkylation	the strongest antiplatelet action was seen in adenine derivatives of 5-methyl- and 5-ethylisatins	*In vitro*	platelet aggregation	[91]

Nanoparticles have a high surface area-to-volume ratio due to their small size. Nanoparticles reflect their bulk material's different electrical, magnetic, and optical characteristics. The synthesis of organic nanoparticles is a popular nanotechnology topic [51]. The study investigated a microbial nano cellulose-ZnO-Ag (CNCs) composite's anticoagulant. When coupled with nanoparticles, bacterial cellulose became an anticoagulant.

Anticoagulant Activity Assay

In silico study

Molecular docking. Molecular docking predicts compound-target protein binding [92]. This method includes algorithms such as molecular simulation, molecular dynamics, and fragment-based methods. Screening and prediction of a drug can be seen from its protein interaction, and a good docking score, glide energy, and glide model can utilize its natural ligand similarities [93]. Some software, receptor proteins, and target compounds used for docking new compounds for coagulants are shown in Table 3. From the screening table, one of the most commonly used software for docking is Autodock 4.2. Additionally, molecular docking was used to better understand the inhibitory actions of newly produced drugs.

The following technique was used to perform cross-docking processes to analyze the appropriateness and representativity of protein structure for virtual screening. Additional receptor and ligan complexes were acquired from the PDB, and their structures were overlaid on the evaluated receptor structure. This yielded quasi-native coordinates of bound ligands compared to the previously examined structure. The RMSD of a docked posture from its quasi-native conformation was used to evaluate drug docking to the receptor structure. A RMSD score of 2 Å was considered satisfactory [72]. Online docking servers include HADDOCK, ClusPro, HDOCK, HEX, NPDock, and MPRDock. However, many web-based docking systems, mainly those using rigid-based docking algorithms, are unable to modify the spatial form and atom coordinates of proteins [11].

Alshehri et al. [93] predicted antithrombotic activity of ten compounds from nature and tested them for docking. The compounds tested using a PASS server, and those showing fibrinolytic properties were caesalpinine c, caesalpinia a, vanillylamine, terpinen-4-ol, dihydrocapsaicin, and 3-carene based on computer-aided molecular modeling and ligand strength validated using binding energies. Cesalpinine c has a high docking score and has more thrombolytic solid action than other drugs [93]. In the study of Ren et al. [94], molecular docking simulations were done with SYBYL 6.9, and Ginsenoside Rg3 targets were estimated with PharmMapper. Cytoscape 3.6.1 was used to develop coagulation disease-component target tissues. Ginseng, red ginseng, notoginseng, *Panax japonicus*, and *Panacis majoris* rhizomes were tested for anticoagulant activity using tissue pharmacology and molecular docking [94]. The binding capability of four compounds from Danshen

Table 3. Target compounds, proteins and software for molecular docking of anticoagulant

Compound	Protein	Docking software	Simulation Software	Ref.
Caesalpinine c, caesalpinine a, vanillylamine, terpinen-4-ol, dihydrocapsaicin and 3-carene	tissue plasminogen activator	Schrödinger-Maestro 12.5	-	[93]
Ginsenoside Rg3	1A4W	SYBYL 6.9	-	[94]
Cryptotanshinone, tanshinone I, dihydrotanshinone I and tanshinone IIA with thrombin or FXa	1DWC	AutoDock 4.2	-	[92]
Eleven designed compounds from dabigatran	1KTS	Surfex-Dock	Amber 14	[56]
Tetrahydropyrazolopyridone derivatives	2P16	Accelrys DS Visualizer 3.0 system	-	[7]
Peptide from ***Porphyra yezoensis***	6B74	pepATTRACT,	GROMACS 5.1.4	[57]
Thirty five novel peptides from casein	2BVR	Discovery Studio 2017, CDOCKER	-	[95]
Chinese patent medicine	2GDE, 2W26	AutoDockTools 4.2.6	-	[96]
Sulfation of citrus pectin	1F9Q, 1JMJ, 4J1Y, 3NXP, 2OK5, 2WXW, 1MD7	Autodock VINA	Amber	[4]
Pharmacophore model derived from dabigatran	1KTS	SYBYL-X 2.0 program	-	[97]
Fluorinated dabigatran analogues	1KTS	SYBYL-X 2.0. package	-	[8]
Anthranilamide derivatives	2W26	FRED	Amber 18	[9]
Epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG)	1DWC	AutoDock 4.2	-	[98]
acylated chitosan sulfate	1SR5	SwissDock web	-	[62]
A highly sulfated 3-linked -arabinan (Ab1)	1PPB, 1TBQ	AutoDock version 4.2	GROMACS 4.0.5	[63]
Heparin tetrasaccharide	3F1S, 3H5C	CLUSPRO	-	[99]
New bi-thiacoumarins derivatives	3KP9	PyRex	-	[64]
Ten new dabigatran derivatives (7a-j)	1KTS	SYBYL-X 2.0	-	[65]
Esculin pentasulfate	1E05	Autodock 4.0	-	[67]
Highly sulfated linear fucan derivatives	1TB6	Autodock 4.0 software	-	[68]
Fluorinated dabigatran derivatives	1KTS	SYBYL 2.0	-	[69]
Calceolarioside B	1PPB	SYBYL8.1 software,	GROMACS	[100]
(2***R***,4***R***,5***S***)- and (2***S***,4***S***,5***R***)-enantiomers of 4-(***tert***-butyl) 2-methyl 5-(4-bromophenyl)-pyrrolidine-2,4-dicarboxylate	2ZC9, 1K1L	AutoDock 4.2.3	-	[10]
Aptamer	1PFX	MPRDock	GROMACS	[11]
Cryptotanshinone (Cry), dihydrotanshinone I (Dih-I) and tanshinone IIA (Dih-I)	1DWC	SYBYL X 2.0	-	[73]
Pyrazolyl piperidine analogs 4(a–h)	2W26	MOE version 2019	-	[74]
1,2,3,4-Tetrahydroquinoline derivatives	4CRC, 1C5P	SOL	-	[72]
Tannic acid	4EKZ	SystemDock	-	[88]

extract (*Saliva miltiorrhiza* Bunge)—cryptotanshinone, tanshinone I, dihydrotanshinone I, and tanshinone IIA—with thrombin or FXa was validated using AutoDock 4.2 [92].

Simulation molecular dynamics. The stability and dynamics of the predicted docked complex are explored using molecular dynamics simulations. The molecular dynamics simulation measures root mean square deviations (RMSD), a radius of gyration (Rg), solvent accessible surface area (SASA), H-bond, and binding free energy. Calculating the backbone RMSD of the free protein and docked complex assessed their dynamic stability from the starting structures across the full journey. The RMSD from the start of the drug-binding region is less fluctuating to the end of the simulation, indicating high stability throughout the simulation of drug binding to the receptor. The stability and cohesiveness of free and complex protein systems during analysis were analyzed in terms of Rg. The Rg is defined as the mass-weighted root of a collection of atoms' average squared distance from the same center of mass. SASA is used to see the surface area of proteins interacting with their molecular solvents. H-bond is a vital intermolecular force that contributes to the stability of complexes between proteins and their binders. After that, the complex's interacting residues' center of mass (COM) interaction distance was also computed. The MM/PBSA method calculated the drug's target-protein receptor binding free energy. Molecular mechanics potential energy (electrostatic and van der Waals interaction) and free energy of solvation (polar and nonpolar solvation energies) are used to estimate binding energy from dynamic trajectories [57].

We utilized RMSF to examine the influence of protein changes during the simulation and looked at fluctuations per residue. The RMSF at the active site is regarded as generally steady between 0 and 1.5 Å. A simulated molecular mechanics/generalized Born surface area (MM/GBSA) analysis examined the complexes' binding free energies. The MM/GBSA technique calculates the binding free energy (ΔG_{bind}) between the ligand (L) and the receptor (R) to produce the complex (R-L) [9]. Khadse et al. [9] did molecular dynamic simulations utilizing Amber18 software examined FXa (GDP: 2W26) in complexation with rivaroxaban and compounds. Submerging the FXa-rivaroxaban complex in a water-truncated TIP3P octahedron neutralized the systems with Na^+ and Cl^- ions. The peptide was modeled using the ff14SB force field [9]. Fernández et al. [63] run all simulations with GROMACS 4.0.5 and the GROMOS96 43a1 force field. The conditions periodic boundaries and the SPC water model dissolve un-complexed human and bovine thrombin in a triclinic box, complexed to non-sulfated arabinan in exosite 2, and complexed to desulfated arabinan in exosite 1. The system was gradually heated from 50 to 310 °K and maintained at 1.0 atm [63].

Biological evaluation

In vitro. The intrinsic route was evaluated using APTT, the extrinsic pathway was assessed using PT, and the degree of fibrin polymerization was evaluated using TT by measuring the duration of fibrin production from fibrinogen. Anti-FXa to evaluate the general pathway of the blood coagulation cascade [14,71,101]. Chen et al. [48] studied blood plasma from healthy adult male rats mixed with 3.8% (v/v, 9:1) sodium citrate solution was tested for anticoagulant action. The platelet-poor plasma (PPP) supernatant was kept at –20 °C. New drugs are dissolved in deionized water. Before the anticoagulant activity test, the drug solution was incubated for 3 min at 37 °C with PPP. The $CaCl_2$ solution was heated and added to the mixture to begin anticoagulant testing. An automatic coagulometer measured clotting time by sensing active APTT, TT, and PT readings [48]. In the presence of new anticoagulant drugs, APTT prolongs but not PT and TT. APTT is specific for the intrinsic blood coagulation pathway, and drugs may interfere with associated clotting factors.

Panax herbs were tested for anticoagulant action *in vitro* using semi-automatic coagulation analysis using four detection devices to assess PT, TT, APTT, and Fib. Total saponin extraction from eight panax samples was diluted in five concentrations in sterile saline. The kit then was properly followed to measure PT, TT, APTT, and Fib. The anticoagulant effect was strongest when RTT, RPT, and RAPTT were greater than zero, and RFib was less than zero. More significant divergence from

zero a higher anticoagulant effect [94]. APTT and PT testing were also carried out by Sun et al. research from volunteer blood or rabbit blood to see the activity of tetrahydropyrazolopyridone derivatives [7].

New medications and standard drugs soluble in physiological buffer were combined with whole blood and incubated for 10 min in a thromboelastography container at 37 °C. The combination was then treated with a thrombin solution to begin whole-blood coagulation. A thromboelastography analyzer tracked clots until they stabilized or lasted an hour. The clot reaction time (R, min), clot kinetics (K, minutes), angle (α, degrees), and maximum amplitude (MA, measured at the widest point) were measured using a fixed plastic pin at 4.75° [49]. The target compound's anticoagulant activity was evaluated *in vitro* using argatroban as a control. Lyophilized human thrombin (national standard), isolated from human blood, was added and incubated for 10 min at 37 °C with the test drug dissolved in DMSO in various dilutions. The technology was then supplemented with a particular fluorogenic thrombin substrate. For 10 min at room temperature, a PerkinElmer Envision microplate reader measures relative fluorescence intensity dynamics. The starting rate of an enzyme reaction is the slope of the linear enzyme dynamics curve at the start. Excitation is 355 nm, and emission is 460 nm. Each well was measured 20 times per 20 s for 10 min. These conditions are used to monitor fluorescence over time. V_{max} slope indicates activity during a kinetic reaction. Calculate the 50% thrombin inhibition (IC_{50}) to determine concentration [8,56].

The thrombin/FXa inhibitory activity of naoxintong capsules extract was assessed using a modified chromogenic substrate and HPLC at 405 nm. Eclipse Plus C18 column (5 m, 2.1 × 150 mm) at 30 °C. For isocratic elution at 4 min, solvent A (water) and solvent B (acetonitrile) were mixed 55:45 (v/v). A 10 µL injection volume and 0.5 mL min^{-1} flow rate were used [96]. FXase test to evaluate FX activation using amidolytic substrate assay due to the presence of exogenous anticoagulants via FIXa. Specific FX and anticoagulant concentrations were incubated with FIXa for 5 min at 25 °C in NaCl, Tris (pH 7.4) containing PL, $CaCl_2$, FVIII, and thrombin vesicles. The reaction was then carried out using ethylenediaminetetraacetic acid (EDTA) while preserving the final concentration of the solution. ELISA plate readers measured FXa levels [62].

Pyrazole dipyridine was tested for anticoagulation against coagulase-positive MRSA using a tube coagulase assay. Sterile brine dissolved rabbit plasma. Plasma was mixed with different test substance doses, grown on MRSA overnight in Eppendorf tubes, and incubated at 37 °C for up to 4 h. Tip the tube to collect formation data and hourly anticoagulant activity. Rabbit plasma and rabbit plasma with MRSA were negative and positive controls, respectively, whereas dabigatran was the benchmark [70]. The plasma sample (100 L) and fractions were incubated in a 37 °C water bath for 1 min before adding calcium chloride and beginning the timer for the PRT test. Glass capillaries gently stirred plasma and fractions every 20 s to detect clots. PRT occurs between calcium chloride addition and the first clot [60].

In vivo. Sun et al. [7] did *in vivo* experimental venous thrombosis was performed using male mice administered orally. Pentobarbital sedated rats and exposed their stomachs. Delicately separated from the surrounding tissue, the inferior vena cava was wrapped in cotton thread under the left renal vein. Two layers of sutures seal the stomach. After 2 h, the stomach was reopened, the vena cava dissected longitudinally, and the thrombus removed. The dry weight of the generated thrombus was evaluated after 24 h at 37 °C [7]. Male Sprague-Dawley rats were studied *in vivo* utilizing a rat tail incision bleeding paradigm. Subcutaneous injections of zoletil were used to anesthetize male rats. The reference sample or novel medicines in physiological buffer were injected intravenously into the tail vein. The mice's tails were cut by 2 mm and submerged in a physiological buffer after 5 min. The student's t-test was used to analyze the bleeding time. A 0.05 P value was significant. Mouse survival probability was calculated using Kaplan-Meier [9,49]. Imran et al. [5] used the bleeding time to observe anticoagulant activity for the new chitosan derivative.

SD mice were injected with the test drug in 0.9% normal saline to block thrombin. After being injected

with anesthetic and fixed supine, the test mice gave free-flowing entire blood on the fourth day. Neck skin was sliced. The right external carotid vein and left carotid artery are separated by one bypass tube. This tube was threaded with a surgical thread, and several topics were injected into the tail vein. The bloodstream is instantly opened for 15 min. The thread is then lifted and examined. By deducting the weight of the surgical suture, the moist weight of the thrombus was estimated. The wet thrombus weight experimental groups' mean and standard deviation values were computed [8,65].

Ex-vivo. Male ICR mice were used for *ex vivo* coagulation testing. An empty group and four groups of mice received the novel drug intragastrically for seven days before blood collection. PPH was made by centrifuging retro-orbital plexus blood in sodium citrate. Anticoagulant activity of APTT, PT, TT, and Fib was tested with a coagulation analyzer [9,60]. Coagulation measurements are used to assess antithrombotic efficacy in experimental rats. Activated clotting time (ACT), clotting rate (CR), platelet function (PF), and clotting time (BCT) were assessed retro-orbitally after blood collection in 3.2% citrate. A coagulation sonoclot and a platelet function analyzer were used for *ex vivo* testing. Representative data exhibiting changes in clot signaling patterns, blood was taken in a cuvette that had already been incubated in the machine, $CaCl_2$ was injected to commence the coagulation cascade, and the data was recorded [67].

■ CONCLUSION

The review has highlighted the promising potential of numerous newly synthesized compounds, derived from both chemical sources and plants, to serve as effective anticoagulants. These findings underscore the importance of ongoing research in this area, as the development of novel anticoagulants holds significant implications for the management of various medical conditions. While the reviewed compounds show promise, it is crucial to emphasize the need for further investigation into their anticoagulant activity and potential toxicity. Future research should focus on conducting in-depth studies to validate their safety and efficacy.

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■ CONFLICT OF INTEREST

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

■ AUTHOR CONTRIBUTIONS

Engrid Juni Astuti wrote the manuscript. Slamet Ibrahim and Muhammad Ali Zulfikar did supervision. Sophi Damayanti develops the concept, supervises, and reviews the manuscript.

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