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Original article

Dual anticoagulant/antiplatelet persulfated small molecules

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ABSTRACT

A new series of persulfated compounds was synthesized and assayed for in vitro anticoagulant and antiplatelet activities, which may be useful in the treatment of both venous and arterial thrombosis. Persulfation of polyphenolic components of wine, coumarins and other structurally diverse small molecules was achieved with triethylamine-sulphur trioxide adduct. The derivatives were highly effective in increasing the APTT, being *trans*-resveratrol 3-*ß*-D-glucopyranoside persulfate (**15**) the most potent ($APTT_2 = 1.5 \times 10^{-4}$ M), and were able to completely block the clotting process at the highest concentration. Compound **15** showed good stability in human plasma and anticoagulation effects in whole blood. *trans*-Resveratrol 3-*ß*-D-glucopyranoside persulfate (**15**) and a series of polysulfated oligoflavonoids (**1**–**4**) also exhibited antiplatelet activity by inhibition of arachidonic acid and ADP-induced platelet aggregation.

1. Introduction

Thrombosis is the most common cause of ischemic cardiovascular disease, such as myocardial infarction and stroke [1]. Currently, available antithrombotic drugs are associated with significant drawbacks that limit their use [2]. The search for new alternative anticoagulant agents with a well defined composition associated to feasible synthesis and fewer secondary effects is a strong challenge to medicinal chemists. The high sulfation level of anticoagulant polysaccharides, like heparin, generates massive negative charge density, thereby introducing the ability to bind to a large number of proteins in the plasma, a probable cause for some of the observed side-effects [3,4]. In order to improve drug bioavailability and reduce heparin side-effects, several small molecules, such as sulfated flavonoids, have been investigated [5–7]. Although simple sulfated flavonoids possess higher hydrophobic nature and lower anionic character than heparin, these derivatives revealed limited anticoagulant potency [5–7]. Therefore, in an effort to improve their potency, we have recently synthesized a series of polysulfated oligoflavonoids that combines some molecular properties of heparin, namely a sulfated saccharidic moiety (Fig. 1). These compounds (**1**–**4**), with both in vitro and in vivo anticoagulant activity, surpassed the anticoagulant activity of sulfated flavonoids [8].

Most of the current approaches in this field are based in the development of selective drugs that modulate targets such as thrombin, factor Xa, or platelet activated receptors. Thrombosis, however, is a complex process involving multiple pathways, often occurring simultaneously associated with inflammatory responses [9]. Inflammatory reactions and continuous activation of the cellular immune system are known to be involved deeply in atherogenesis, and oxidative stress as well as moderate hyperhomocysteinemia are argued to represent major triggers of cardiovascular disease progression [10]. It can be assure that cardiovascular diseases are related with a multifactorial pathogenesis. Using drugs that modulate the activity of a single protein target the probability to efficiently stop the progression of the cardiovascular pathological scenario is low [11], and the strategy “multi target-direct-ligands” must be considered in drug discovery in this area [11].

Based on the above considerations, we planned to investigate sulfated small molecules that could interact in the complex molecular pathways involved in the atherothrombotic disease. Following this principle, polyphenolic components of wine were considered attractive building blocks for molecular modification by sulfation due to their cardioprotective actions. Several epidemiological

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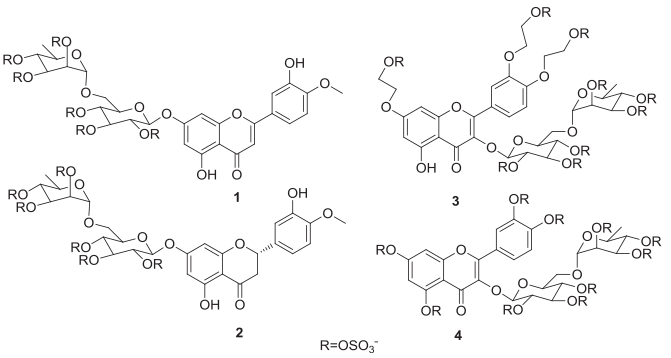


Fig. 1. Polysulfated oligoflavonoids (**1**–**4**) with anticoagulant activity [8].

studies [12] and particularly the "French Paradox" [13] have demonstrated that mortality from coronary heart disease can be reduced by moderate consumption of alcoholic beverages, especially red wine. The protective effects of wine has been attributed to a complex mixture of bioactive compounds that are predominantly phenolic in nature, with strong antioxidant and anti-inflammatory properties [14]. These include flavonols such as myricetin, kaempferol, and quercetin, the flavan-3-ol monomers catechin and epicatechin, the oligomeric and polymeric flavan-3-ols or proanthocyanidins, various highly coloured anthocyanins, an assortment of phenolic acids (gallic acid, caftaric acid, caffeic acid, *p*-coumaric acid), and the stilbene resveratrol [15]. *trans*-Resveratrol is present in red wine at concentrations up to 7.2 mg/L [16,17] and has been suggested to possess cancer chemopreventive, antioxidative, antiplatelet, and cardioprotective properties [15,18,19]. Five resveratrol sulfates, including 3,4′,5-trisulfate, 3,4′-disulfate, 3,5-disulfate, 4′-sulfate, and 3-sulfate were recently synthesized. Although they were less potent than resveratrol, they retained some of resveratrol antioxidant and anti-inflammatory activities [20].

Thus, we postulated that the sulfated derivatives of polyphenolic components of wine **5**–**8** (Fig. 2) would be expected to retain inherent activities of their precursors and additionally would lead to attractive anticoagulant agents. Based on the success of sulfated oligoflavonoids (**1**–**4**), two polyphenolic components of wine with a saccharidic moiety were selected: *trans*-resveratrol 3-*ß*-D-glucopyranoside (**7**) and chlorogenic acid (**8**).

Another class of naturally occurring bioactive substances, coumarins, were also used for molecular modification by sulfation. Coumarins are particularly variable in structure, and their biological activities are influenced by various types of substitutions in their scaffold [21]. Thus, two coumarins with a saccharidic moiety, 4-methyl 7-hydroxycoumarin 7-*ß*-D-glucopyranoside (**9**) and 6,7-dihydroxycoumarin 6-*ß*-D-glucopyranoside (esculin, **10**) were also submitted to sulfation. Considering the association of some compounds with randomly activities associated to cardiovascular scenario, the following small molecules were also selected for sulfation: ascorbic acid (**11**), a non-phenolic non-glucosidic compound, and 2-(hydroxymethyl)phenyl *ß*-D-glucopyranoside (D-(−)-salicin, **12**), a non-phenolic glucosidic compound (Fig. 2).

In this work, the synthesis and the in vitro anticoagulant and antiplatelet profiles of eight structurally diverse persulfated small molecules are described. In vitro clotting time assays in human plasma were used to screen the anticoagulant activity of the synthesized compounds and, for the most promising molecules, a complete anticoagulant profile in human whole blood was further obtained using thromboelastography (TEG). In order to understand the mechanism of action of the persulfated compounds, enzyme inhibition assays were performed against factor Xa, thrombin, prothrombin, protein C and antithrombin III. The platelet aggregation was also investigated in whole blood with the platelet function analyzer (PFA-100) and with the new impedance aggregometer Multiplate.

The obtained dual acting anticoagulant/antiplatelet molecules are expected to lead to a novel approach in the prevention and treatment of cardiovascular diseases.

2. Chemistry

Sulfation of two simple phenolic acids, gallic acid (**5**) and ellagic acid (**6**), two hydroxycinnamic acids, *trans*-resveratrol 3-*ß*-D-glucopyranoside (**7**) and chlorogenic acid (**8**), two coumarins, 4-methyl 7-hydroxycoumarin 7-*ß*-D-glucopyranoside (**9**) and esculin (**10**), ascorbic acid (**11**) and D-(−)-salicin (**12**) was achieved with triethylamine-sulfur trioxide adduct (2–8 equiv/OH) in dimethylacetamide, at 65 °C (Scheme 1).

Following the reaction conditions (Scheme 1), gallic acid persulfate (**13**), ellagic acid persulfate (**14**), *trans*-resveratrol 3-*ß*-D-glucopyranoside persulfate (**15**), chlorogenic acid persulfate (**16**), 4-methyl 7-hydroxycoumarin 7-*ß*-D-glucopyranoside persulfate (**17**), esculin persulfate (**18**), ascorbic acid persulfate (**19**), and salicin persulfate (**20**) were successfully obtained in moderate yields (Scheme 1). Compounds **13**–**19** are described for the first time. Salicin persulfate (**20**) was previously obtained by synthesis [22]. To solve the problem associated with the separation of persulfated compounds, highly soluble in water, from the water soluble impurities, purification procedures involving dialysis for compounds **16** and **18** and SPE cartridges for compound **14** were necessary (see experimental protocols).

3. Results and discussion

3.1. Clotting assays

The in vitro anticoagulant activity was initially screened for all sulfated and non-sulfated compounds (**5**–**20**) in human plasma by

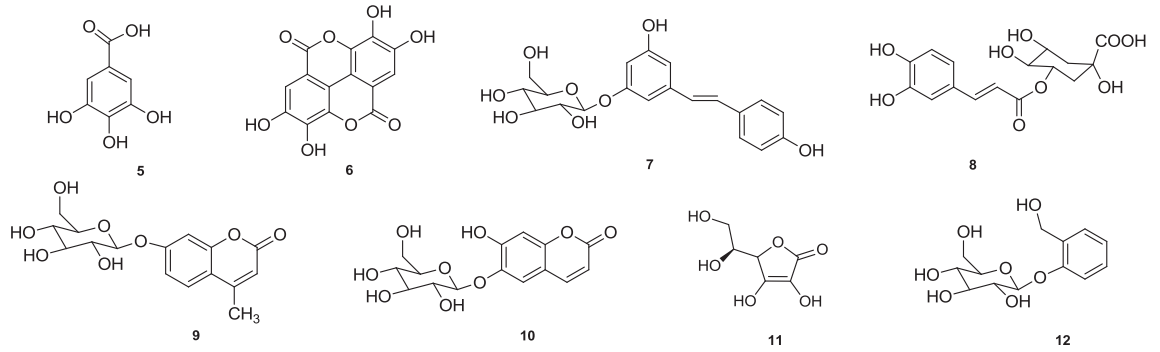
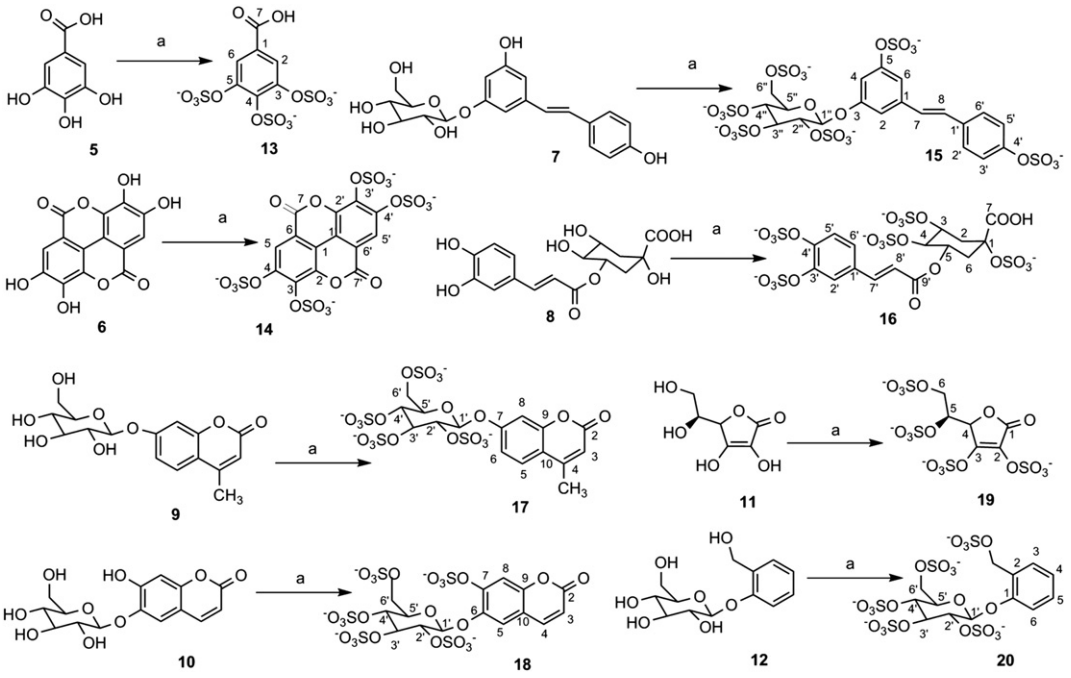


Fig. 2. Structurally diverse small molecules (**5**–**12**) selected for sulfation in this study.



Scheme 1. Reagents and conditions for the synthesized compounds **13**–**20**. (a) triethylamine-sulfur trioxide adduct (2 equiv/OH for compounds **5** and **11**; 4 equiv/OH for compounds **6** and **7**; 6 equiv/OH for compounds **8** and **10**; 8 equiv/OH for compound **9** and **12**), DMA, 65 °C. The numbering used concerns the NMR assignments.

the three classical clotting times, activated partial thromboplastin (APTT), prothrombin (PT), and thrombin time (TT). The non-sulfated parent compounds **5**–**12** were also considered in order to explore the influence of the sulfate group on the anticoagulant activity.

The results for the sulfated derivatives (**13**–**20**) on APTT, PT, and TT are summarized in Fig. 3 and Table 1. Fig. 3 represents the dose-dependent effects for the persulfated derivatives **13** and **15**–**20** on the three clotting tests. Compound **14** was only tested at 5×10^{-4} M since it was not obtained in sufficient amounts. In Table 1 the concentration needed to double the coagulation times ($APTT_2$, PT_2, TT_2) was calculated for the most potent compounds. The non-sulfated compounds **5**–**12** were inactive in all clotting time assays (data not shown; at the concentration tested, 5×10^{-3} M, no significant difference was observed between the compound and the control). The investigated sulfated compounds **13** and **15**–**20** showed anticoagulant properties in a dose-dependent manner (Fig. 3).

Prolongation of APTT was observed for all sulfated compounds (**13**–**20**). The compounds were able to completely block this clot formation pathway at high concentrations (Fig. 3, Table 1), except compounds **13** and **16**. *trans*-Resveratrol 3-*ß*-D-glucopyranoside persulfate (**15**), esculin persulfate (**18**), and ascorbic acid persulfate (**19**) were the most potent compounds in prolonging APTT ($APTT_2 = 1.5 \times 10^{-4}$, 3.1×10^{-4}, and 3.2×10^{-4} M, respectively).

Although prolongation of PT was observed for compounds **15** and **17**–**20**, none of these were able to completely inhibit this clotting pathway. Only *trans*-resveratrol 3-*ß*-D-glucopyranoside persulfate (**15**) was able to double the PT (Table 1) and the sulfated compounds **17**–**20** only increased the PT approximately 1.5 times, at the highest concentration tested (5×10^{-3} M). Compounds **13**, **14**, and **16** were not active at the highest concentration tested (5×10^{-4} M for compound **14**, 5×10^{-3} M for compounds **13** and **16**).

The TT was only sensitive to the presence of *trans*-resveratrol 3-*ß*-D-glucopyranoside persulfate (**15**) (Fig. 3, Table 1).

In summary, all sulfated compounds (**13**–**20**) prolonged APTT more than PT and only compound **15** influenced the TT pathway.

Some structure–activity relationships can be observed. Firstly, *trans*-resveratrol 3-*ß*-D-glucopyranoside persulfate (**15**), with the

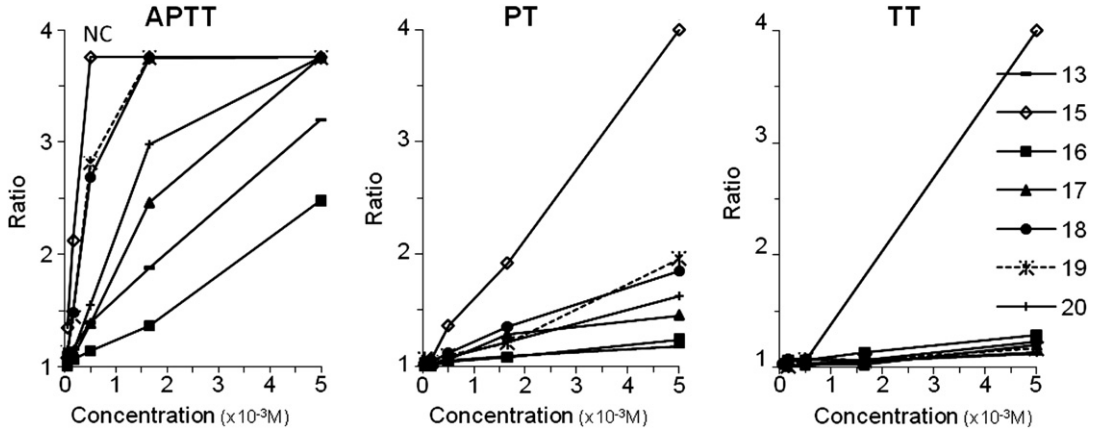


Fig. 3. Effects of persulfated derivatives (**13**, **15**–**20**) on APTT, PT and TT clotting assays using human pooled plasma, expressed as ratio of clotting time in the presence/absence of compound. Data points represent the average of three experiments performed in duplicate with a standard deviation <10%. nc = no coagulation.

Table 1
Effects[a] of sulfated derivatives **13**–**20** in the clotting assays.

Compd	APTT total inhibition	$APTT_2$[b]	PT_2[b]	TT_2[b]
13	–	1.960	na	na
14	0.50	nd	na	na
15	0.50	0.146	1.770	1.947
16	–	3.659	na	na
17	5	1.175	>5	na
18	1.65	0.309	>5	na
19	1.65	0.315	>5	na
20	5	0.899	>5	na

($P > 0.05$).

[a] Values in concentrations expressed in 10^{-3} M represent the average of three independent experiments with a standard deviation < 10%; positive control, heparin: $APTT_2$ = 0.4 U/mL, PT_2 = 1.9 U/mL and TT_2 = 0.2 U/mL.

[b] The concentration required to double clotting time. nd = not determined. na = not active at 5×10^{-3} M.

highest degree of sulfation (six sulfate groups), was the most potent in prolonging the clotting times, which highlight the importance of the number of sulfate groups for the anticoagulant activity. This was also observed when comparing both coumarins derivatives. Comparing these results with the earlier obtained for polysulfated flavonosides (**1**–**4**, Fig. 1) [8], it is interesting to note that the hexasulfated derivative of *trans*-resveratrol (**15**, $APTT_2 = 1.46 \times 10^{-4}$ M) was even more potent than hexasulfated diosmin (**1**, $APTT_2 = 4.35 \times 10^{-4}$ M) and hexasulfated hesperidin (**2**, $APTT_2 = 4.13 \times 10^{-4}$ M). Also, when comparing different classes of compounds with the same number of sulfates, namely compounds with four sulfate groups (**14**, **17**, and **19**) it can be noted that the chemical class of the sulfated scaffold seems to influence the prolongation of APTT. The importance of the persulfated-*O*-glycoside moiety on the anticoagulant activity could be highlighted when comparing the weak anticoagulant effect showed by sulfated compound **16** with compounds **18** and **20**, all with five sulfate groups. The weak anticoagulant effect of compound **16** could also be related to the presence of carboxyl group, since COOH-containing compounds (**13** and **16**) were the less active compounds.

As the three clotting assays record interactions at different stages on the coagulation process (Scheme 2, **A**), preliminary information about the mode of action could be obtained. The APTT identifies the time interval required for clot formation in response to a non-physiological stimulus that leads to primary activation of the intrinsic pathway [23]. APTT is used as a screening test to identify acquired or inherited deficiencies or inhibitors of factors VIII, IX, and XI [23]. Reduced activation of fibrinogen, prothrombin, or factors V and X (common pathway) can also prolong the APTT [23]. APTT is sensitive to heparin anticoagulant therapy, which exerts its therapeutic effect by acceleration of antithrombin III, inactivation of factor Xa, and thrombin [24]. The PT assay measures the activity of coagulation factors of the extrinsic pathway and is therefore a screening tool of the activities of fibrinogen, prothrombin and factors V, VII, and X [23]. PT is the assay used to monitor the oral anticoagulants, since they lower the levels of prothrombin, VII, and X [24]. The TT screens the formation of fibrin from fibrinogen after the addition of known amounts of thrombin to the plasma sample [23]. As the obtained persulfated compounds (**13**–**20**) prolonged APTT and PT (APTT more than PT), their mechanism of action may be related to the common pathway of the coagulation cascade (factors X, V, prothrombin, and fibrinogen) and compound **15**, as affects the TT, must be also implicated in the last step, the conversion of fibrin to fibrinogen by thrombin.

Thus, to investigate their anticoagulant mechanism of action, several targets such as thrombin, factor Xa, antithrombin III, prothrombin, and protein C were selected for further studies and a thromboelastography (TEG) analysis was planned to assess the effects of sulfated compounds (**13**, **15**–**20**) on all blood components.

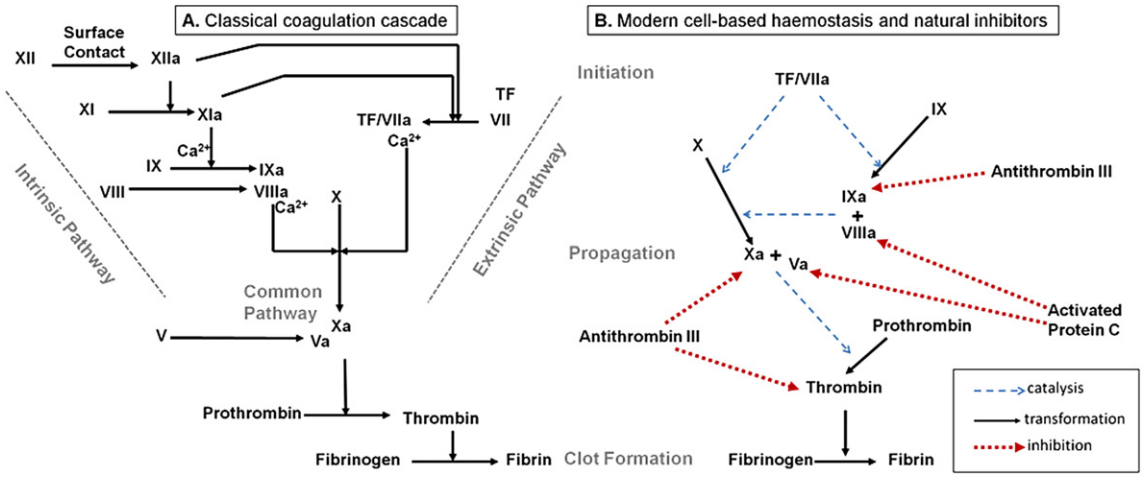
3.2. Enzyme inhibition studies

Compounds **13** and **15**–**20** were firstly evaluated for selectivity against the critical enzymes targeted by current anticoagulant therapy: thrombin, factor Xa, and antithrombin III. Inhibition of thrombin and factor Xa, in the presence and in absence of antithrombin III, was followed by spectrophotometric determination of the product formed after amide bond cleavage of the chromogenic substrates Chromozym TH and CBS 31.39, respectively and the initial rate compared with that obtained in the absence of the compound. Reference inhibitors EDTA (direct factor Xa inhibitor), LMWH (antithrombin III/factor Xa activator), and PPACK (direct thrombin inhibitor) were also tested in the same conditions.

None of the tested compounds (**13** and **15**–**20**) showed any relevant influence on the activity of the proteinases tested, at 1×10^{-3} M, highlighting that another mechanism of action could be implicated in the antithrombotic effect of these compounds.

3.3. Protein C and prothrombin levels

To assess if the anticoagulant effect of sulfated polyphenolic compounds **13** and **15**–**20** could interfere with the protein C pathway,



Scheme 2. Coagulation models.

the determination of activated protein C (APC) in citrated human plasma was evaluated by a functional test. Protein C was activated by a specific activator derived from the venom of *Agkistrodon c. contortrix* and the quantity of enzyme thus formed was measured by its amidasic activity on the synthetic substrate CBS 42.46. Additionally, the sulfated oligoflavonoids **1**–**4** (Fig. 1) were also investigated.

The APC and prothrombin levels in the presence of a single concentration (1×10^{-3} M) of sulfated compounds **1**–**4**, **13**, and **15**–**20** are shown in Table 2. Fig. 4 represents the dose-dependent effect of compounds **3**, **4**, and **15** on APC and prothrombin levels.

At 1×10^{-3} M, compounds **1**, **2**, **13**, **16**–**20** had no significant ($P > 0.05$) influence in the protein C activity (Table 2). At the same concentration, compounds **3**, **4**, and **15** decreased the APC to pathological levels (less than 50%, Table 2). Nevertheless, human plasma after in vitro exposure to compounds **3**, **4**, and **15**, at the concentration that lead to approximately 40–60% of APC inhibition, also showed a deeply deficiency in prothrombin levels (Table 2, Fig. 4).

trans-Resveratrol 3-β-glucoside persulfate (**15**) was the most potent in prolonging the clotting times and was selected for this study due to the cardiovascular effects of *trans*-resveratrol. The mechanism of action of *trans*-resveratrol, although is not fully understood [25], seems to be related to the inhibition of the induction of TF expression in endothelial cells and mononuclear cells [26] and/or to preventing homocysteine accumulation in the blood [27]. High levels of homocysteine (hyperhomocysteinemia) increase the risk of atherosclerosis and thrombosis, although the mechanism by which hyperhomocysteinemia might contribute to in vivo atherogenesis and thrombogenesis are scarcely understood. In vitro studies showed that homocysteine, among other effects, inhibits both protein C activation and the activity of APC [24]. The function of APC is to inactivate factor Va and factor VIIIa (Scheme 2, **B**) [28]. The first step in this process is the activation of thrombomodulin by thrombin. Subsequently, protein C combines with thrombomodulin in order to produce APC. APC combines with protein S on the surface of a platelet [28] and then degrade factor Va and factor VIIIa. Apparently the interaction of homocysteine with cysteine residues of factor V, interferes with the proteolytic action of APC on factor Va, resulting in inhibition of APC activity. Thus, *trans*-resveratrol by preventing homocysteine accumulation in the blood, is described to prevent the inhibition of the natural anticoagulant APC [24]. However, in contrast to *trans*-resveratrol, the persulfated 3-β-glucoside **15** and compounds **3** and **4** inhibited de APC at the highest concentration tested, and consequently the risk of thrombosis could be suspected at 1×10^{-3} M. However, inhibition of APC is also described with heparin and oral anticoagulants [29–32] and the effectiveness of these anticoagulants, despite the fact that they severely depress protein C levels, is the observation that prothrombin levels are depressed. The efficacy of APC as an anticoagulant seems to be much greater at low prothrombin levels (near those observed in stably anticoagulated patients) than at normal levels [29]. The low levels of prothrombin at the concentration that lead to APC inhibition, lead us to rule out the risk of thrombosis in the presence of compounds **3**, **4**, and **15**. Furthermore, these results allowed to clarify the TT prolongation effects observed in the presence of compound **3**, **4**, and **15**, which were not found for the others compounds (**1**, **2**, **13**, **16**–**20**). Prothrombin is converted to thrombin by factor Xa and with low levels of thrombin the conversion of fibrin to fibrinogen is reduced and the TT is prolonged.

Table 2
Effects[a] of sulfated compounds **1**–**4**, **13**, and **15**–**20** (1×10^{-3} M) on activated protein C (APC) and prothrombin levels.

Human plasma	APC levels	Prothrombin levels
Normal plasma (STApreciclot Plus I)	102 ± 3.3 [99–135]	99 ± 5.6 [80–108]
Pathological plasma (STApreciclot Plus II)	38 ± 4.1 [32–44]	36.7 ± 2.1 [30–42]
Pool (H_2O)	101.5 ± 2.1	96.7 ± 5.2
1	87 ± 1.2	54.0 ± 5.7*
2	85 ± 1.1	47.5 ± 6.4*
3	38.5 ± 0.7*	10.9 ± 0.8*
4	28.5 ± 0.7*	9.0 ± 0.5*
13	101 ± 1.7	61.5 ± 2.1*
15	50.5 ± 0.7*	12.0 ± 0.9*
16	95 ± 0.3	82.0 ± 2.8
17	97 ± 0.1	64.5 ± 0.7*
18	87 ± 1.0	64.5 ± 3.5*
19	94 ± 0.9	91.5 ± 12.0
20	93 ± 0.7	66.0 ± 2.8*

*$P < 0.05$.
[a] Results expressed as percentage. Each value represents the average ± SD of three independent experiments done in duplicate.

3.4. Thromboelastography

Separation of the coagulation cascade into the intrinsic, extrinsic, and common pathways is now known to be an in vitro phenomenon (Scheme 2, **A**) [23]. In vivo, tissue factor released from damaged endothelial cells combines with factor VIIa (traditionally considered extrinsic), and then activates factors IX and X (Scheme 2, **B**). Factor IXa combines with factor VIIIa (traditionally considered intrinsic) and also activates factor X. Factor Xa combines with factor Va and converts prothrombin to thrombin, which then cleaves fibrinogen into fibrin [33]. In fact, the intrinsic and extrinsic pathways are not isolated entities in vivo. Classical clotting tests (section 3.1.) are performed in citrated plasma and provide information only about one isolated segment of the coagulation system (secondary haemostasis). The clot quality and the impact of platelets on coagulation are not assessed by these assays. In contrast, thromboelastography (TEG) is performed in whole blood and the clot is induced under a low shear environment resembling an in vivo sluggish venous flow [33]. TEG allows the acquisition of quantitative information about the kinetics of clot formation and growth as well as the clot strength and stability attained by clots [33]. Thus, to have a global picture of the anticoagulant effect of sulfated molecules **13** and **15**–**20**, TEG analyses were carried out.

The anticoagulant effects of sulfated compounds **15**, **18**, and **19** in whole human blood were evaluated at the double of the $APTT_2$ concentration (3.12×10^{-4} M for compound **15** and 6.25×10^{-4} M for compounds **18** and **19**) (Table 3). Additionally, compounds **13**, **16**, **17**, and **20** were tested at the same concentration of 1×10^{-3} M. Modified thromboelastograms were obtained by adding to the whole blood an intrinsic activator (InTEM) or an extrinsic activator (ExTEM). It was also possible to eliminate platelet contribution from the TEG sample and study the fibrin part of the clot by adding cytochalasin D and Ca^{2+} (FibTEM). In Fig. 5, the TEG representative tracings of persulfated compound **15** are illustrated. The parameters obtained from the InTEM thromboelastogram were R, the period of time from initiation of the test to the initial fibrin formation (clotting time); K, the time from beginning of clot formation until the amplitude of thromboelastogram reaches 20 mm (clotting formation time); $\propto$ angle, the acceleration/kinetics of fibrin build up and cross-linking (clot formation and growth); and MA, the maximum clot firmness/maximum amplitude of the clot (Table 3).

No alterations on thromboelastogram were observed in the presence of compounds **13**, **16**, **17**, and **20**, at 1×10^{-3} M (data not shown). Additionally, compound **19** showed no anticoagulant effect in whole blood at the concentration that have increased 4 fold the APTT in plasma (data not shown). In the presence of compounds **15** and **18**, abnormal thromboelastograms were obtained when an intrinsic activator was added in InTEM test (Table 3), an effect

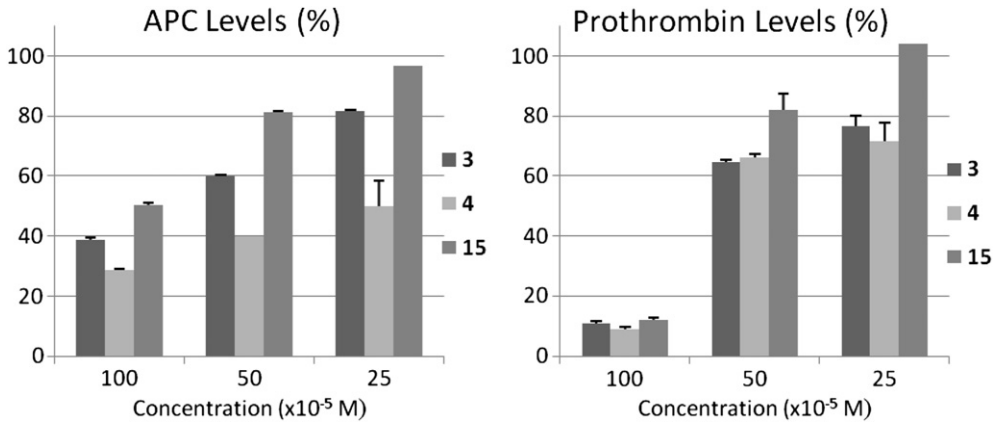


Fig. 4. Dose-dependent inhibitory effects of compounds **3**, **4**, and **15** on APC and prothrombin levels.

similar to the observed when a factor deficiency is present (anticoagulants or haemophilia) [34]. Compared to control values, compound **18** increased only the clotting time (R), while compound **15** increased also the clotting formation time (K), and decreases ∝ and MA (Table 3). This indicates that the kinetics of fibrin polymerization and networking, and the strength of the formed clot, that is highly dependent of platelets, is significantly retarded/decreased by the presence of sulfated compound **15**, while compound **18** is only affecting the clotting factors. Nevertheless, normal thromboelastograms were obtained in the presence of compound **15** when the platelet-specific effect on MA of TEG tracings is eliminated (FibTEM test) (Fig. 5). These results allowed the conclusion that an action on platelets would also be implicated in the mode of action of compound **15**.

3.5. Platelet aggregation studies

Some sulfated flavonoids found in literature were studied by their effects on platelet function in the presence of different agonists [35]. Quercetin 3-acetyl-3′,4′,7-*O*-trisulfate, quercetin 3,3′,4′,7-*O*-tetrasulfate [36], and genistein-4′-*O*-sulfate [37] inhibited collagen-induced platelet aggregation while quercetin 7-*O*-sulfate [38] and quercetin 4′,7-*O*-disulfate [39] exhibited the ability to inhibit thrombin-induced platelet aggregation. Moreover, resveratrol has been described as an in vitro inhibitor of the collagen-induced platelet aggregation as well as an in vivo ADP-induced platelet aggregation inhibitor [18,40].

Based on the above considerations and on the thromboelastograms obtained, platelet aggregation in the presence of sulfated compounds **15**, **18**, and **19** was evaluated with the Platelet Function Analyzer (PFA-100). PFA-100 measures the platelet function in the whole blood instead of plasma, the last used in the previous described methods for sulfated small molecules [36–39]. Whole blood is the physiological environment where platelet function takes place in vivo, and the use of whole blood for in vitro assays also eliminates the need for time-consuming centrifugation steps. Additionally, the PFA-100 provides a rapid quantitative measurement of platelet adhesion and aggregation in whole blood flowing through a small aperture under high shear conditions [41]. In PFA-100, platelets are activated by a collagen-coated membrane and an additional agonist, adenosine 5′-diphosphate (ADP) or epinephrine (EPI). The time required for platelets to occlude the central aperture in the membrane is reported as the closure time.

Table 3
Effects[a] of persulfated compounds **15** (3.12×10^{-4} M), **18**, and **19** (6.25×10^{-4} M) in the whole human blood on the parameters obtained from InTEM modified thromboelastography (TEG).

Whole blood	Thromboelastography InTEM test					
	R(s)[b]	K(s)[c]	∝(°)[d]	A10 (mm)[e]	A20 (mm)[f]	MA (mm)[g]
Reference range	100–240	30–110	70–83	44–66	50–71	50–72
Blood (saline)	188 ± 21	82 ± 3	77 ± 3	51 ± 2	58 ± 3	57 ± 1
15	447 ± 76*	136 ± 19*	64 ± 3*	41 ± 5	49 ± 4	50 ± 2
18	340 ± 29*	95 ± 9	71 ± 1	49 ± 2	53 ± 1	53 ± 1
19	205 ± 4	84 ± 2	74 ± 1	51 ± 1	56 ± 1	56 ± 1

*$P < 0.05$.
[a] Each value represents the average ± standard error of the mean (SEM) of three independent experiments done in duplicate.
[b] R, clotting time expressed in seconds (s).
[c] K, clotting formation time expressed in seconds (s).
[d] ∝ angle expressed in degrees (°).
[e] A10, amplitude expressed in mm at 10 min.
[f] A20, amplitude expressed in mm at 20 min.
[g] MA, maximum amplitude expressed in mm.

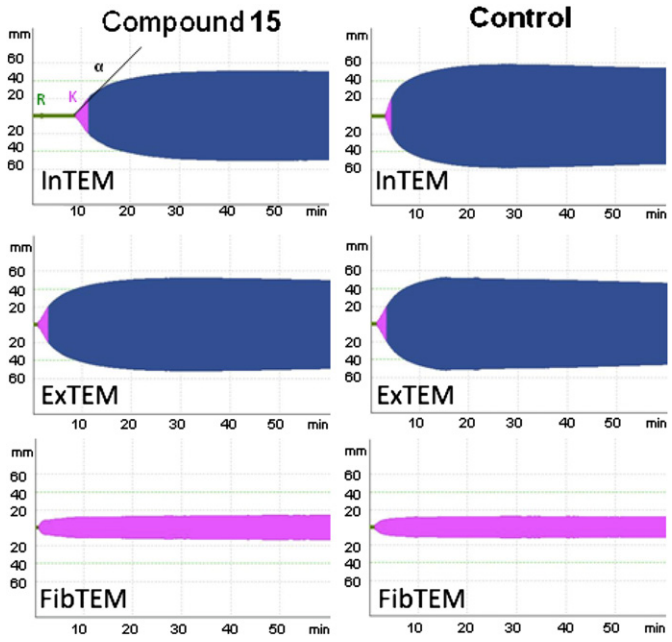


Fig. 5. Thromboelastography (TEG) representative tracings of persulfated compound 15 (3.12×10^{-4} M).

Following, specific effects of compounds **1**–**4**, **15**, **18**, and **19** on platelet thrombin receptor, arachidonic acid cascade and ADP receptors were evaluated using the Multiplate analyser. The Multiplate analyser is an advancement of impedance aggregometry [42]. It assesses platelet function in whole blood by the attachment of thrombocytes onto metal electrodes, leading to a change of the electrical conductivity (or impedance), which is continuously recorded. While PFA-100 analysis provides a global evaluation of platelet function, Multiplate provides a more specific evaluation [43]. For example, the PFA-100 method uses collagen and EPI as agonists, neither of which is specific for ciclooxigenase-1 (COX-1) activity, the target of acetylsalicylic acid. In contrast, in the Multiplate system, platelets can be activated by multiple specific agonists such as thrombin receptor-activating peptide (TRAP test), arachidonic acid (ASPI test), and adenosine diphosphate (ADP test). In recent years, TRAP test has been used to detect GpIIb/IIIa antagonists, as abciximab, tirofiban, and eptifiban, while ASPI test has been used to control the acetylsalicylic acid antiaggregant effect, and ADP test to control clopidogrel, prasugrel, and cangrelor antiaggregant agents [44,45].

The platelet aggregation in the presence of sulfated compounds **1**–**4**, **15**, **18**, and **19** are summarized in Table 4. For comparison proposes, the closure times obtained for sulfated flavonosides **1**–**4** [8] were also included in Table 4. The Multiplate-ASPI representative tracings for compounds **1**–**4** and **15** are illustrated in Fig. 6.

Closure times have been shown not to vary with heparin administration [46], while acetylsalicylic acid has been shown to increase the EPI closure time [47], and clopidogrel the ADP closure time [46]. Compound **15** prolonged both the ADP and EPI closure times (Table 4). Since both closure times were increased, we hypothesized that compound **15** could cause a platelet dysfunction. The addition of compounds **1**–**4** and **15**, into the blood led to a reduced aggregation response in both ASPI test and ADP test (Table 4). ASPI test specifically measures the activation by arachidonic acid, the substrate of COX-1. COX forms thromboxane A2 which is a potent platelet activator. When the platelet cyclooxygenase is blocked, the formation of thromboxane A2 is inhibited and no alteration or minor platelet activation is recorded. Thus, the decreased area under the aggregation curve (AUC) obtained in the ASPI test in the presence of compounds **1**–**4** and **15** (Fig. 6, Table 4) showed their ability to act as platelet cyclooxygenase inhibitors. The AUC obtained at 6.25×10^{-4} M for compounds **1**–**4** and **15** was similar to the one obtained with acetylsalicylic acid (same conditions) (Fig. 6). Compounds **1**–**4** and **15** also inhibited aggregation responses in the ADP test, which may indicate an interaction also on the ADP receptors, as thienopyridine derivatives.

These results are in agreement with the mechanism of action hypothesized for quercetin 3,3′,4′,7-*O*-tetrasulfate as an inhibitor of thromboxane A2 receptor [36]. Additionally, in vitro COX-1 and COX-2 inhibitory activities of sulfated resveratrol metabolites were also reported [20] with the 3-sulfate and the 4′-sulfate metabolites inhibiting COX-1 (3.60 and 5.55 μM, respectively) with IC_{50} values comparable to resveratrol (6.65 μM). Molecules such as compounds **1**–**4** and **15**, combining in a single molecule anticoagulant and platelet antiaggregatory activities could be very promising in the prevention and treatment of cardiovascular diseases [48]. In the last five years, dual anticoagulant/antiplatelet agents have been described. Examples are the pyridoxine and pyridoxal analogues which can inhibit thrombin as well as TRAP and ADP-induced platelet aggregation [48], the 1,4-benzoxazin-3(4*H*)-one derivatives which are thrombin inhibitors and also displayed fibrinogen receptor antagonists properties [49,50], and the fluorinated benzyloxyphenyl piperidine-4-carboxamides as dual inhibitors of factor Xa and platelet aggregation [51]. Nevertheless, molecules which simultaneously target both the antiplatelet and anticoagulant pathways have not yet achieved clinical development. A dual agent should be advantageous, due to expected less complex pharmacokinetics, a likely lower incidence of side-effects, and less demanding clinical studies. We expect that dual acting anticoagulant/antiplatelet molecules such as *trans*-resveratrol 3-*β*-D-glucopyranoside persulfate (**15**) and sulfated oligoflavonoids (**1**–**4**) could lead to a new therapeutic strategy in the prevention and treatment of thromboembolic events.

Table 4
Antiaggregant effects[a] of sulfated compounds **1**–**4**, **15**, **18**, and **19** (6.25×10^{-4} M) on whole human blood evaluated with PFA-100 and Multiplate analysers.

Whole blood	PFA-100 Closure time (s)		Multiplate AUC (U)[b]		
	Collagen-ADP	Collagen-EPI	TRAP test[c]	ASPI test[d]	ADP test
Reference values	71–118	94–193	84–128	71–115	57–113
Blood (saline)	119 ± 8	168 ± 5	106[f]	72[f]	76[f]
ASA[e]	nd	nd	nd	23 ± 0.3*	nd
1	162 ± 16* [8]	171 ± 3 [8]	109[f]	34 ± 22*	20 ± 5*
2	139 ± 7* [8]	167 ± 17 [8]	120[f]	15*[f]	15*[f]
3	204 ± 44* [8]	>300* [8]	84[f]	33*[f]	33*[f]
4	189 ± 13*[,g] [8]	244 ± 45*[,g] [8]	95[f]	21.5 ± 9.5*	23 ± 6*
15	212*[,f]	273*[,f]	102[f]	40 ± 1.7*	17.3 ± 2.8*
18	nd	nd	89[f]	98[f]	98[f]
19	106[f]	121[f]	110[f]	90.5 ± 0.5	68 ± 1

*$P < 0.05$.
[a] Each value represents the average ± SEM of three independent experiments done in duplicate.
[b] area under the aggregation curve (AUC) expressed in U (1U = 10 AU*min).
[c] thrombin receptor-activating peptide test.
[d] arachidonic acid test.
[e] Acetylsalicylic acid at 6.25×10^{-4} M positive control.
[f] Values performed in duplicate.
[g] Determined at 3.12×10^{-4} M nd = not determined.

3.6. Stability studies

Human plasma stability is an important parameter to anticipate and assess in preclinical studies. Thus, stability studies in human plasma were performed for compound **15**. For that propose an ion-pairing reverse-phase high-performance liquid chromatography (IP-RP-HPLC) method was developed (see experimental protocols). A 3 h time point was selected to cover the time period of an in vivo administration. The isolation of compound **15** from plasma samples was performed by the addition of cold acetonitrile for deproteinization of plasma samples. To avoid insolubilization of compound **15** in this step, ion-pairing of compound **15** was previous performed by addition of tetrabutylammonium (TBA) buffer (10 mM potassium dihydrogen phosphate and 25 mM TBA, adjusted to pH 8 with TEA) to the plasma samples. Following, quantitative determination of compound **15** was performed for each time (time zero and 3 h) by IP-RP-HPLC. Compound **15** was found to be stable for 3 h in human plasma, as the concentration of at least 95% of the initial one was detected. The stability of compound **15**, and the already described stability of compounds **1** and **4** [8], in human plasma, warranty that the anticoagulant/antiaggregant activity cannot be attributed to decomposition products, such as desulfonated by-products, or non-sulfated parent molecules.

These results, added to the putative in vivo efficacy obtained for sulfate derivatives **1** and **4** [8], reflect their drug-like properties. Additionally, in vivo studies suggested that **1** and **4** will not induce acute hepatic toxicity. Furthermore, the effects of the derivatives **1**–**4**, **13**, **15**, **17**, **18**, and **20** on in vitro growth of three representative human tumor cell lines - MCF-7 ER(+) (breast adenocarcinoma), NCI-H460 (non small cell lung cancer), and A375-C5 (melanoma) - suggested that these compounds do not affect cell viability (no

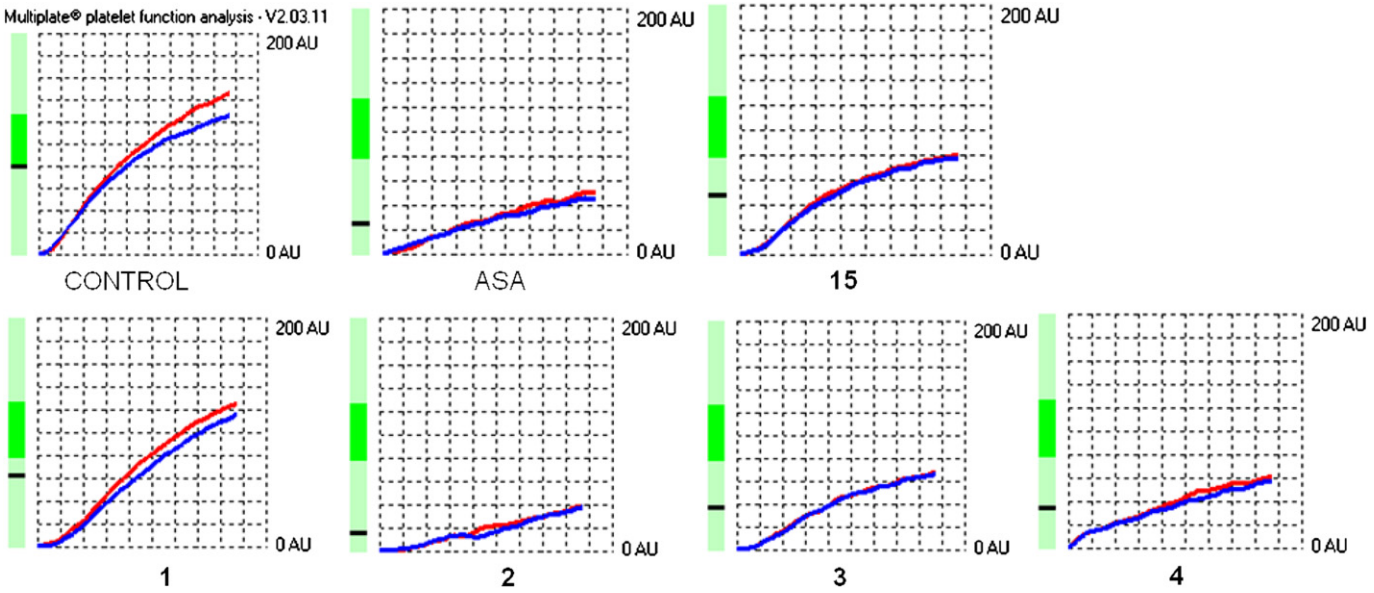


Fig. 6. Typical Multiplate tracing in ASPI test: during a measurement period of 6 min the change in electrical impedance is calculated from the mean values of the two curves. Control, saline; ASA, acetylsalicylic acid (final concentration, 6.25×10^{-4} M); **1**–**4**, **15**, sulfated compounds (final concentration, 6.25×10^{-4} M).

alterations in the viable cells number were observed during 48 h of treatment at 1.5×10^{-4} M; data not shown). The overall results lead us to rule out the putative toxicity of these polysulfated scaffolds.

4. Conclusion

In this work, dual acting modulators for thrombosis were developed. *trans*-Resveratrol 3-ß-D-glucopyranoside persulfate (**15**) and a series of sulfated oligoflavonoids (**1**–**4**), combining anticoagulant and antiplatelet effects into a single molecule, are expected to prevent and treat both, venous and arterial, thrombosis, with less complex pharmacokinetics and side-effects. Furthermore, as these polysulfated molecules derived from chemical molecular modifications of compounds that have already been used in humans as drugs and/or nutraceutics, we belief they will produce safe therapeutic agents. This series of small molecules represent the first step in the direction of a new generation of cardiovascular agents.

5. Experimental

5.1. Chemistry

Triethylamine-sulfur trioxide adduct (S 5139) and chlorogenic acid (**8**, 25700) were purchased from Fluka (Spain), gallic acid (**5**, D 3525), ellagic acid (**6**, 45140), salicin (**12**, S 0625), 4-methyl 7-hydroxycoumarin 7-*ß*-D-glucopyranoside (**9**, M 3633), esculin (**10**, E 8250), ascorbic acid (**11**, A 5960), and *trans*-resveratrol 3-*ß*-D-glucopyranoside (**7**, 572691), were purchased from Sigma–Aldrich (Spain). The solvents used were products *pro analysis* or HPLC grade of the firms Sigma–Aldrich and Fluka. Spectra/Por Dialysis membrane MWCO: 1000 was purchased from Spectrum Labs, Inc (California, US). TLC was performed using Polygram cel 400 UV_{254} 0.1 mm (Macherey–Nagel, Germany) (BuOH–CH_3COOH–H_2O 4:2:6 and 5:2:3) and Merck silica gel 60 (GF254) plates ($CHCl_3$:MeOH 9:1). Compounds were visually detected by absorbance at 254 nm and/or 365 nm. Melting points were obtained in a Köfler microscope and are uncorrected. IR spectra were measured on an ATI Mattson Genesis series FTIR (software: WinFirst v.2.10) spectrophotometer in KBr microplates (cm^{-1}).

^1H and ^{13}C NMR spectra were taken in DMSO-d_6 at room temperature, on Bruker Avance 300 and 500 instruments (300.13 or 500.13 MHz for ^1H and 75.47 MHz for ^{13}C). Chemical shifts are expressed in δ (ppm) values relative to tetramethylsilane (TMS) as an internal reference. Coupling constants are reported in hertz (Hz). ^{13}C NMR assignments were made by 2D HSQC and HMBC experiments (long-range C, H coupling constants were optimized to 7 and 1 Hz).

The high resolution mass spectra were measured on an APEX III Mass Spectrometer, recorded as ESI (Electrospray) mode in Centro de Apoio Científico e Tecnolóxico á Investigation (C.A.C.T.I., University of Vigo, Spain).

HPLC chromatographic conditions: the HPLC analyses were carried out on a System SMI Pump Series II (Gloucester, UK) equipped with a Rheodyne 7125 injector fitted with a 20 µL loop, a TSP-UV6000LP detector, and a Chromquest for Windows NT integrator and using a C-18 Nucleosil column (5 µm, 250 × 4.6 mm I.D.), from Macherey–Nagel (Düren, Germany); acetonitrile was of HPLC grade from Merck. HPLC ultrapure water was generated by a Milli-Q system (Millipore, Bedford, MA, USA); the mobile phases were degassed for 15 min in an ultrasonic bath before use; the mobile phase used was 10 mM of phosphate buffer with 25 mM of TBA (adjusted to pH 8 with TEA) and acetonitrile (55:45) at a constant flow rate of 1.0 mL/min.

5.1.1. General procedure for sulfation

To a solution of each selected compound **5**–**12** (2 mmol) in DMA (15 mL), triethylamine-sulfur trioxide adduct (2–8 equiv/OH) was added and the suspension was heated at 65 °C for 24 h. The mixture was poured into acetone (150 mL) under basic conditions (few mL of triethylamine) and left at 4 °C for 24 h. The crude oil formed was washed with acetone and ether, and dissolved in aqueous solution of 30% sodium acetate (5 mL). Generally, the suspension was added drop-wise in ethanol to precipitate the sodium salt of the sulfated derivative.

*5.1.1.1. 3,4,5-Trisulfate benzoic acid (**13**).* The precipitate obtained according to the general procedure corresponded to the sodium salt of compound **13** (yield 36%). White solid, mp >250 °C (ethanol). IR (KBr)υmax: 1570, 1258, 1014, 857. ^1H NMR (DMSO-d_6, 300.13 MHz)

δ: 7.85 (2H, s, H-2 and H-6). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 169.0 (C-7), 146.4 (2C, C-2 and C-6), 136.2 (C-4), 121.2 (C-1), 115.9 (2C, C-3 and C-5). HRMS (ESI$^+$): *m/z* calcd for $C_7H_3O_{14}S_3Na_4$ 498.82702, found 498.82710.

*5.1.1.2. 2,3,7,8-Tetrasulfate chromeno [5,4,3-cde]chromene-5,10-dione (**14**).* The precipitate obtained according to the general procedure corresponded to an inorganic salt (monitored by IR analysis). Treatment of the filtrate by removing the solvent under reduced pressure afforded a crude oil. Aqueous solution of 30% of sodium acetate was added to the crude oil to afford sodium salts. An impure brown solid precipitated from ethanol. The solid was purified by solid phase extraction with a cation exchange cartridge Discovery® (DSC-SCX, GraceResolv), with a sulfonic acid moiety following the steps: condition with methanol (3 mL); equilibrate with water (3 mL); load sample solution in water (1 mL); wash with 5% methanol in water (8 mL); wash with 2% acetic acid in methanol/water; elution with 2% NH_4OH in methanol/water (15 mL). The NH_4OH fractions were collected and the solvent evaporated under reduced pressure to afford compound **14** (yield 4%). Pallid-brown solid, mp >250 °C. IR (KBr)υmax: 1610, 1256, 1014, 856. ^{1}H NMR (DMSO-d_6, 300.13 MHz) δ: 7.87 (2H, s, H-5 and H-5′). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 160.0 (C-7 and C-7′), 147.5 (C-4 and C-4′), 145.0 (C-3 and C-3′), 131.0 (C-2 and C-2′), 116.6 (C-1 and C-1′), 116.5 (C-5 and C-5′), 99.4 (C-6 and C-6′).

*5.1.1.3. (2R,3R,4S,5S,6R)-2-[3-sulfate-5-[(E)-2-(phenyl-4-sulfate)ethenyl] phenoxy]-6-(methylsulfate)oxane-3,4,5-triol (**15**).* The precipitate obtained according to the general procedure corresponded to an inorganic salt (monitored by IR analysis). The filtrate was alkalinized with bicarbonate and extracted with CH_2Cl_2 (3 × 30 mL). The aqueous phases were collected, washed with CH_2Cl_2, and the solvent was removed by evaporation under reduced pressure, affording the sodium salt of compound **15** (yield 12%). Pallid-yellow solid, mp 209–210 °C (water). IR (KBr)υmax: 1636, 1250, 1033, 845. ^{1}H NMR (DMSO-d_6, 300.13 MHz) δ: 7.58 (2H, d, *J* = 8.5 Hz, H-2′ and H-6′), 7.17–6.93 (6H, m, H-3′, H-5′, H-2, H-6, H-7, H-8), 6.64 (1H, s, H-4), 5.41 (1H, d, *J* = 7.3 Hz, H-1″), 4.66–3.85 (6H, m, H-6″a, H-2″, H-3″, H-4″, H-5″, H-6″b). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 158.0 (C-3), 154.4 (C-5), 138.6 (C-1), 131.9 (C-1′), 128.4 (C-8), 127.3 (C-2′, 6′), 126.9 (C-7), 120.3 (C-3′, 5′), 119.1 (C-4′), 112.1 (C-6), 109.0 (C-2), 108.5 (C-4), 98.8 (C-1″), 80.5 (C-3″), 78.1 (C-5″), 74.7 (C-2″), 71.3 (C-4″), 67.1 (C-6″). HRMS (ESI$^+$): *m/z* calcd for $C_{20}H_{16}O_{26}S_6Na_7$ 1024.75324, found 1024.75408.

*5.1.1.4. (1S,3R,4R,5R)-3-[(E)-3-(phenyl-3,4-disulfate)prop-2-enoyl]oxy-1,4,5-trisulfate-cyclohexane-1-carboxylic acid (**16**).* An oily product was obtained according to the general procedure and was further purified from other salts (monitored by IR analysis) using a Spectra/Por 6 Regenerated Cellulose MWCO1,000 (yield 87%). Oily product. IR (KBr)υmax: 1711, 1260, 1014, 840. ^{1}H NMR (DMSO-d_6, 500.13 MHz) δ: 7.78 (1H, d, *J* = 2.0 Hz, H-2′), 7.47 (1H, d, *J* = 15.9 Hz, H-7′), 7.60 (1H, d, *J* = 8.5 Hz, H-5′), 7.28 (1H, dd, *J* = 8.5 and 2.0 Hz, H-6′), 6.12 (1H, d, *J* = 15.9 Hz, H-8′), 5.61 (1H, m, H-5), 4.78 (1H, d, *J* = 11.8 Hz, H-4), 4.33 (1H, m, H-3), 2.84 (1H, d, *J* = 9.2 Hz, H-6a), 2.30 (1H, d, *J* = 14.1 Hz, H-2a), 2.03 (1H, d, *J* = 10.8 Hz, H-2b), 1.99 (1H, d, *J* = 12.2 Hz, H-6b). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 172.8 (C-7), 165.1 (C-9′), 146.4 (C-4′), 144.7 (C-7′), 143.6 (C-3′), 127.7 (C-1′), 123.6 (C-6′), 119.6 (C-5′), 119.2 (C-2′), 115.7 (C-8′), 78.7 (C-1), 72.3 (C-3), 70.1 (C-4), 68.8 (C-5), 34.0 (C-6), 32.0 (C-2). HRMS (ESI$^+$): *m/z* calcd for $C_{16}H_{13}O_{24}S_5Na_6$ 886.7786, found 886.77810.

*5.1.1.5. 4-Methyl-7-[(2S,3R,4S,5S,6R)-3,4,5-trisulfate-6-(methylsulfate) oxan-2-yl] oxychromen-2-one (**17**).* The precipitate obtained according to the general procedure corresponded to the sodium salt of compound **17** (yield 47%). pallid-yellow solid, mp >340 °C (ethanol). IR (KBr)υmax: 1617, 1257, 1032, 847. ^{1}H NMR (DMSO-d_6, 300.13 MHz) δ: 7.73 (1H, d, *J* = 8.7 Hz, H-8), 7.04 (1H, dd, *J* = 8.8 and 1.5 Hz, H-6), 6.93 (1H, d, *J* = 1.5 Hz, H-5), 6.24 (1H, s, H-3), 5.57 (1H, d, *J* = 2.9 Hz, H-1′), 4.59 (2H, m, H-2′ and H-4′), 4.42 (1H, m, H3′), 4.00–3.88 (2H, m, H-6′a/H-6′b), 1.16 (3H, s, 4-C*H*$_3$). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 160.2 (C-2), 160.2 (C-7), 154.4 (C-9), 153.6 (C-9/C-4), 113.6 (C-6), 103.6 (C-5), 111.8 (C-3), 114.3 (C10), 126.7 (C-8), 98.4 (C-1′), 75.2 (C-5′), 74.1 (C-2′/C4′), 73.9 (C-2′/C4′), 70.9 (C-3′), 67.1 (C-6′), 18.3 (4-*C*H$_3$). HRMS (ESI$^+$): *m/z* calcd. for $C_{16}H_{14}O_{20}S_4Na_5$ 768.8450, found 768.84443.

*5.1.1.6. 7-Sulfate-6-[(2S,3R,4S,5S,6R)-3,4,5-trisulfate-6-(methylsulfate) oxan-2-yl]oxychromen-2-one (**18**).* The precipitate obtained according to the general procedure was further purified from other salts (monitored by IR analysis) using a Spectra/Por 6 Regenerated Cellulose MWCO1,000 (yield 26%). Orange solid, mp >340 °C (water). IR (KBr)υmax: 1619, 1258, 1031, 846. ^{1}H NMR (DMSO-d_6, 300.13 MHz) δ: 8.04 (1H, d, *J* = 9.5 Hz, H-4), 7.55 (2H, s, H-5/H-8), 6.31 (1H, d, *J* = 9.5 Hz, H-3), 5.34 (1H, d, *J* = 3.4 Hz, H-1′), 4.73 (2H, m, H-2′ and H-4′), 4.44–4.41 (1H, m, H-5′), 4.19 (1H, d, *J* = 2.5 Hz, H-6′a), 4.15–4.09 (1H, m, H-3′), 3.74 (1H, t, *J* = 9.8 Hz, H-6′b). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 160.5 (C-2), 148.7 (C-9), 146.4 (C-7), 144.8 (C-4), 143.8 (C-6), 114.0 (C-5), 113.6 (C-3), 113.1 (C-10), 106.6 (C-8), 99.7 (C-1′), 75.7 (C-4′), 75.3 (C-2′), 74.8 (C-3′), 71.7 (C-5′), 67.0 (C-6′). HRMS (ESI$^+$): *m/z* calcd for $C_{15}H_{11}O_{24}S_5Na_6$ 872.76245, found 872.76243.

*5.1.1.7. (5R)-[(1S)-ethyl-1,2-disulfate]-3,4-disulfate furan-2(5H)-one (**19**).* The precipitate obtained according to the general procedure corresponded to an inorganic salt (monitored by IR analysis). Treatment of the filtrate by removing the solvent under reduced pressure afforded a crude oil. Aqueous solution of 30% of sodium acetate was added to the crude oil and added drop-wise in ethanol to precipitate the sodium salt of compound **19** (yield 7%). Brown solid, mp 160–162 °C (ethanol). IR (KBr)υmax: 1613, 1256, 1030, 843. ^{1}H NMR (DMSO-d_6, 300.13 MHz) δ: 4.52 (1H, q, H-5), 4.29 (1H, m, H-4), 4.14 (1H, q, H-6′), 3.73 (1H, t, H-6′′). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 173.1 (C-1), 154.2 (C-3), 95.7 (C-2), 92.4 (C-4), 70.2 (C-5), 63.1 (C-6).

*5.1.1.8. (2R,3S,4S,5R,6R)-2-(methyl-O-sulfate)-6-[2-(methyl-O-sulfate) phenoxy]oxane-3,4,5-triol (**20**) [22].* The precipitate obtained according to the general procedure corresponded to an inorganic salt (monitored by IR analysis). The filtrate was alkalinized with bicarbonate and extracted with CH_2Cl_2 (3 × 30 mL). The aqueoses phases were collected, washed with CH_2Cl_2, and the solvent was removed by evaporation under reduced pressure, affording sulfated compound **20** (yield 71%). Pallid-yellow solid, mp >250 °C (water). IR (KBr)υmax: 1631, 1240, 1062, 796. ^{1}H NMR (DMSO-d_6, 300.13 MHz) δ: 7.38 (1H, d, *J* = 7.7 Hz, H-3), 7.23 (1H, t, *J* = 7.6 Hz, H-5), 6.99 (1H, d, *J* = 7.5 Hz, H-4), 6.93 (1H, t, *J* = 8.1 Hz, H-6), 5.27 (1H, d, *J* = 4.9 Hz, H-1′), 4.91 (2H, s, C*H*$_2$OH), 4.65–4.27 (4H, m, H-2′, H-3′, H-4′, H-5′), 3.99 (1H, m, H-6'a), 3.83 (1H, m, H-6'b). ^{13}C NMR (DMSO-d_6, 75.47 MHz) δ: 153.7 (C-1), 127.9 (C-2), 127.3 (C-5), 127.0 (C-3), 121.4 (C-4), 113.1 (C-6), 98.1 (C-1′), 76.2–71.5 (C-2′, C-3′, C-4′, C-5′), 67.3 (C-6′), 62.7 (*C*H$_2$OH). HRMS (ESI$^+$): *m/z* calcd for $C_{13}H_{13}O_{22}S_5Na_6$ 818.78827, found 818.78862.

5.2. Biological activity

5.2.1. Clotting assays

Human blood was obtain from 10 healthy donors aged between 25 and 45 years old without history of bleeding or thrombosis and who had not taken any medication known to affect blood coagulation and platelet function for 2 weeks. Venous blood was obtained

and transferred to a plastic tube. Nine volumes of blood were decalcified with one volume of 3.8% sodium citrate solution. Blood was centrifuged for 20 min at 2400 g and the pooled plasma was stored at −20 °C until use. Compounds **5**–**7** were dissolved in water with 10% DMSO and compounds **8**–**20** were dissolved in water. The final concentration of sulfated compounds **13** and **15**–**20** in these assays ranged from 5×10^{-3} M to 0.05×10^{-3} M and only a single concentration was tested for sulfated compound **14** (5×10^{-4} M) and non-sulfated compounds **5**–**12** (5×10^{-3} M). Heparin was used as positive control at concentration range 0.1–10 UI/mL. In the control group water with 10% DMSO for compounds **5**–**7** or water for compounds **8**–**20** was used. APTT, PT, and TT tests were performed using an ACL100 coagulometer (IZASA, Portugal). The following commercial kits were used: 49735320 (IZASA, Portugal) for the APTT, 20002900 (IZASA, Portugal) for the PT and 9758515 (IZASA, Portugal) for the TT. The assays were carried out according to reference. The clotting times were recorded in seconds (s). Each measurement was performed in duplicate and repeated three times on different days ($n = 6$). Coagulation time prolonging ratio was calculated comparing the clotting time in the presence of each tested compound with that obtained when water was used instead of test compound. The concentration required to double the clotting time was calculated from linear regression analysis of each individual concentration-response curve.

5.2.2. Chromogenic substrate hydrolysis assays

Human factor Xa (27 nkat/mL) and human thrombin (137 NIH/mL) were obtained from Diagnostica Stago (Roche, Portugal). Bovine antithrombin III was obtained from Sigma–Aldrich (Germany) (50 UI/mL). The chromogenic substrates used were Chromozym TH (Tosyl-Gly-Pro-Arg-*p*-nitroaniline(*p*NA), Roche Applied Science) for thrombin and CBS 31.39 (CH_3SO_2-D-Leu-Gly-Arg-*p*NA, AcOH; Diagnostica Stago) for factor Xa. Stock solutions of the enzymes were obtained by restitution according to the manufacture instructions and assay solutions were prepared by fresh dilution with the assay buffer. The assay buffer was 50 mM Tris/HCl at pH 8.3, containing 227 mM NaCl and 0.1% (w/v) bovine serum albumin (BSA, Sigma–Aldrich) instead of PEG-8000. The final enzyme concentrations were 0.5 U/mL, 0.06 U/mL, and 1 U/mL for factor Xa, thrombin, and antithrombin III, respectively. The final substrate concentrations in the reactions were 178 μM and 125 μM (in water) for Chromozym TH and CBS 31.39, respectively. Under the experimental conditions, less than 10% of the substrate was consumed in all assays [ϵ_{405nm} (*p*-nitroaniline) = 10.4 $mmol^{-1}$ L cm^{-1}]. The chromogenic assays were carried out according to reference [8]. Absorbance was calculated by subtracting the absorbance at each point with the absorbance measured without the enzyme. The control was performed using water in place of the test compound. The initial slope of the absorbance curve in the presence of the test compound was compared to that of the control to obtain the percentage of inhibition. Measurements were carried out in duplicate and repeated at least three times on different days ($n = 6$).

5.2.3. Protein C activity

PC activity was measured after addition of compound solution (30 μL, saline) to citrated human plasma (300 μL) using a chromogenic assay (STA protein C chromogen 6007935, Diagnostica Stago, Roche, Portugal). Protein C was activated by a specific activator derived from the venom of *Agkistrodon c. contortrix* (100 μL) and the quantity of enzyme thus formed was measured by its amidasic activity on the synthetic substrate CBS 42.46 (200 μL). Calibration curve was obtained with a linearity range 3–150% (% $C = 546.5365$*DO/min-3.1556; $r^2 = 1.000$) with STA Unicalibrator (608035). Normal plasma (STA PreciClot Plus I, Diagnostica Stago, Roche, Portugal) and pathological plasma (STA PreciClot Plus II, Diagnostica Stago, Roche, Portugal) were used for checking the accuracy and reproducibility of results.

5.2.4. Prothrombin factor levels

After the addition of compound solution or water (50 μL) citrated pooled human plasma (100 μL) was diluted with factor diluent solution (350 μL, IZASA, Portugal). The clotting time (PT) was determined after mixing the plasma with factor II deficient plasma. High and low calibration curves were prepared with calibrated plasma using dilutions automatically performed by the instrument (ACL100 coagulometer, IZASA, Portugal). Linearity range 1.56–6.25% ($r^2 = 0.999$) for low calibration curve and linearity range 25–100% ($r^2 = 0.999$) for high calibration curve. Normal plasma (STA PreciClot Plus I, Diagnostica Stago, Roche, Portugal) and pathological plasma (STA PreciClot Plus II, Diagnostica Stago, Roche, Portugal) were used for checking the accuracy and reproducibility of results.

5.2.5. Thromboelastography

Blood samples from healthy donors were collected into siliconized vacumtainer tubes containing 3.8% trisodium citrate such that a ratio of citrate/whole blood of 1:9 (v/v) was maintained. Sulfated compounds were dissolved in saline and diluted in the whole blood (1:16) to a final concentration of 3.12×10^{-4} M (compound **15**), 6.25×10^{-4} M (compound **18** and **19**) and 1×10^{-3} M (compounds **13**, **16**, **17**, and **20**). In the control group saline was added to the whole blood (1:16). The TEG assay was performed according to reference [8].

5.2.6. Platelet aggregation studies

5.2.6.1. Platelet function closure times. Blood samples from healthy donors were collected into tubes containing hirudin with a final concentration of 25 μg/mL and analysed within the period of 0.5–1 h after collection. In vitro platelet function was evaluated using a PFA-100 device (Dade Behring, Frankfurt, Germany) according to reference [8].

5.2.6.2. Multiplate electrical impedance aggregometry. Blood samples from healthy donors were collected into tubes containing hirudin with a final concentration of 25 μg/mL and analysed within the period of 0.5–1 h after collection. Compound solution (125 μL, saline) was added to whole blood (1875 μL) to a final concentration of 6.25×10^{-4} M. The assays were carried out according to the respective instructions of the manufacturer (Multiplate, Dynabyte, Munich, Germany): 300 μL of whole blood at room temperature were added to 300 μL of pre-heated saline at 37 °C; after an incubation time of 180 s, 20 μL of the selected agonist solution (Dynabyte, Munich, Germany) was added, giving final concentrations of thrombin receptor-activating peptide (TRAP-6) at 32 μM (TRAP test), arachidonic acid at 0.5 mM (ASPI test), or adenosine diphosphate (ADP) at 6.5 μM (ADP test). The instrument continuously measures the change in resistance during 6 min, which is proportional to the amount of platelets adhering to the electrodes and transforms it to arbitrary "aggregation units" (AU); these are plotted against time (min) and give the area under the curve (AUC) in U (1U = 10 AU*min), calculated from the mean values of the two curves. The analysis was accepted when the difference between the two curves is <20%.

5.2.7. Stability studies

The plasma stability of compound **15**, was performed at 25 μM (final concentration) in human plasma previously diluted 1:1 with phosphate buffer (PBS, pH 7.4). Three independent samples, plus respective blank and controls, were analyzed for each time (time zero and 180 min). Incubations were performed in eppendorfs, on

a bath shaker, at 37 °C for 180 min. After the addition of tetrabutylammonium (TBA) buffer (10 mM potassium dihydrogen phosphate and 25 mM TBA, adjusted to pH 8 with TEA), the reaction was quenched with 4 × cold acetonitrile (HPLC grade), followed immediately by mixing and centrifugation during 15 min at 14000 rpm. The time zero samples were quenched immediately after the sample was added to plasma. After filtration (milipore), the supernatant was evaporated and the residue dissolved with mobile-phase (50 μM final concentration), and transferred to HPLC vials.

5.3. Statistical analysis

Data were tested for statistical significance by non-parametric two-tailed Mann–Whitney test using GraphPad Prism 5 software. A value of $P < 0.05$ was considered significant.

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References

[1] J.F. Viles-Gonzalez, V. Fuster, J.J. Badimon, Atherothrombosis: a widespread disease with unpredictable and life-threatening consequences, Eur. Heart J. 25 (2004) 1197–1207.
[2] J. Fareed, D.A. Hoppensteadt, D. Fareed, M. Demir, R. Wahi, M. Clarke, C. Adiguzel, R. Bick, Survival of heparins, oral anticoagulants, and aspirin after the year 2010, Semin. Thromb. Hemost. 34 (2008) 58–73.
[3] J. Hirsh, T.E. Warkentin, S.G. Shaughnessy, S.S. Anand, J.L. Halperin, R. Raschke, C. Granger, E.M. Ohman, J.E. Dalen, Heparin and low-molecular-weight heparin - mechanisms of action, pharmacokinetics, dosing, monitoring, efficacy, and safety, Chest 119 (2001) 64S–94S.
[4] B.H. Chong, Heparin-induced thrombocytopenia, J. Thromb. Haemost. 1 (2003) 1471–1478.
[5] H.A. Guglielmone, A.M. Agnese, S.C.N. Montoya, J.L. Cabrera, Anticoagulant effect and action mechanism of sulphated flavonoids from Flaveria bidentis, Thromb. Res. 105 (2002) 183–188.
[6] G.T. Gunnarsson, U.R. Desai, Interaction of designed sulfated flavanoids with antithrombin: lessons on the design of organic activators, J. Med. Chem. 45 (2002) 4460–4470.
[7] G.T. Gunnarsson, M. Riaz, J. Adams, U.R. Desai, Synthesis of per-sulfated flavonoids using 2,2,2-trichloro ethyl protecting group and their factor Xa inhibition potential, Bioorg. Med. Chem. 13 (2005) 1783–1789.
[8] M. Correia-da-Silva, E. Sousa, B. Duarte, F. Marques, F. Carvalho, L.M. Cunha-Ribeiro, M.M.M. Pinto, Flavonoids with an oligo-polysulfated moiety: a new class of anticoagulant agents, J. Med. Chem. 54 (2011) 95–106.
[9] D.D. Wagner, New links between inflammation and thrombosis, Arterioscler. Thromb. Vasc. Biol. 25 (2005) 1321–1324.
[10] P. Libby, Y. Okamoto, V.Z. Rocha, E. Folco, Inflammation in Atherosclerosis: transition from theory to practice, Circ. J. 74 (2010) 213–220.
[11] R. Morphy, Z. Rankovic, Designed multiple ligands. An emerging drug discovery paradigm, J. Med. Chem. 48 (2005) 6523–6543.
[12] M. Gronbaek, Epidemiologic evidence for the cardioprotective effects associated with consumption of alcoholic beverages, Pathophysiology 10 (2004) 83–92.
[13] S. Renaud, M. Delorgeril, Wine, alcohol, platelets, and the French Paradox for coronary heart-disease, Lancet 339 (1992) 1523–1526.
[14] E.N. Frankel, J.B. German, J.E. Kinsella, E. Parks, J. Kanner, Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine, Lancet 341 (1993) 454–457.
[15] L. Brown, P.A. Kroon, D.K. Das, S. Das, A. Tosaki, V. Chan, M.V. Singer, P. Feick, The biological responses to resveratrol and other polyphenols from alcoholic beverages, alcohol, Clin. Exp. Res. 33 (2009) 1513–1523.
[16] A. Bertelli, A.A.E. Bertelli, A. Gozzini, L. Giovannini, Plasma and tissue resveratrol concentrations and pharmacological activity, Drugs Exp. Clin. Res. 24 (1998) 133–138.
[17] R. Vidavalur, H. Otani, P.K. Singal, N. Maulik, Significance of wine and resveratrol in cardiovascular disease: french paradox revisited, Exp. Clin. Cardiol. 11 (2006) 217–225.
[18] E.G. Fan, L.J. Zhang, S. Jiang, Y.H. Bai, Beneficial effects of resveratrol on atherosclerosis, J. Med. Food 11 (2008) 610–614.
[19] S.V. Penumathsa, N. Maulik, Resveratrol: a promising agent in promoting cardioprotection against coronary heart disease, Can. J. Physiol. Pharmacol. 87 (2009) 275–286.
[20] J. Hoshino, E.J. Park, T.P. Kondratyuk, L. Marler, J.M. Pezzuto, R.B. van Breemen, S.Y. Mo, Y.C. Li, M. Cushman, Selective synthesis and biological evaluation of sulfate-conjugated resveratrol metabolites, J. Med. Chem. 53 (2010) 5033–5043.
[21] R.D.H. Murray, J. Mendez, S.A. Brown (Eds.), The Natural Coumarins. Ocurrence, Chemistry and Biochemistry, John Wiley and Sons, Chichester, 1982.
[22] A. Raghuraman, M. Riaz, M. Hindle, U.R. Desai, Rapid and efficient microwave-assisted synthesis of highly sulfated organic scaffolds, Tetrahedron Lett. 48 (2007) 6754–6758.
[23] T. Ruttmann, Coagulation for the clinician, S. Afr. J. Surg. 44 (2006) 22–37.
[24] S.P. Bajaj, J.H. Joist, New insights into how blood clots: Implications for the use of APTT and PT as coagulation screening tests and in monitoring of anticoagulant therapy, Semin. Thromb. Hemost. 25 (1999) 407–418.
[25] U. Stervbo, O. Vang, C. Bonnesen, A review of the content of the putative chemopreventive phytoalexin resveratrol in red wine, Food Chem. 101 (2007) 449–457.
[26] U.R. Pendurthi, J.T. Williams, L.V.M. Rao, Resveratrol, a polyphenolic compound found in wine, inhibits tissue factor expression in vascular cells - A possible mechanism for the cardiovascular benefits associated with moderate consumption of wine, Arterioscler. Thromb. Vasc. Biol. 19 (1999) 419–426.
[27] K. Schroecksnadel, C. Winkler, B. Wirleitner, H. Schennach, G. Weiss, D. Fuchs, Anti-inflammatory compound resveratrol suppresses homocysteine formation in stimulated human peripheral blood mononuclear cells in vitro, Clin. Chem. Lab. Med. 43 (2005) 1084–1088.
[28] C.T. Esmon, The protein C pathway, Chest 124 (2003) 26S–32S.
[29] M.D. Smirnov, O. Safa, N.L. Esmon, C.T. Esmon, Inhibition of activated protein C anticoagulant activity by prothrombin, Blood 94 (1999) 3839–3846.
[30] J. Hirsh, J.E. Dalen, D.R. Anderson, L. Poller, H. Bussey, J. Ansell, D. Deykin, Oral anticoagulants: mechanism of action, clinical effectiveness, and optimal therapeutic range, Chest 119 (2001) 8S–21S.
[31] R. De Cristofaro, E. De Candia, R. Landolfi, Effect of high- and low-molecular-weight heparins on thrombin-thrombomodulin interaction and protein C activation, Circulation 98 (1998) 1297–1301.
[32] B. Furie, C.F. Diuguid, M. Jacobs, D.L. Diuguid, B.C. Furie, Randomized prospective trial comparing the native prothrombin antigen with the prothrombin time for monitoring oral anticoagulant-therapy, Blood 75 (1990) 344–349.
[33] S.M. Donahue, C.M. Otto, Thromboelastography: a tool for measuring hypercoagulability, hypocoagulability, and fibrinolysis, J. Vet. Emerg. Crit. Care 15 (2005) 9–16.
[34] T. Fenvyivesi, M. Lakner, I. Träger, I. Jörg, C. Giese, J. Harenberg, Effect of direct thrombin inhibitors, low molecular weight heparins dalteparin and enoxaparin and of the heparinoid danaparoid on the rotation thrombelastometry method (ROTEM), Haemostaseologie 25 (2005) 138.
[35] M.E. Sousa, M. Correia-da-Silva, M.M.M. Pinto, in: G. Brahmachari (Ed.), Natural Products: Chemistry, Biochemistry and Pharmacology, Narosa Publishing House, New Delhi, 2008, pp. 392–416.
[36] H.A. Guglielmone, A.M. Agnese, S.C. Nunez-Montoya, J.L. Cabrera, Inhibitory effects of sulphated flavonoids isolated from Flaveria bidentis on platelet aggregation, Thromb. Res. 115 (2005) 495–502.
[37] G. Rimbach, P.D. Weinberg, S. de Pascual-Teresa, M.G. Alonso, B.A. Ewins, R. Turner, A.M. Minihane, N. Botting, B. Fairley, S. Matsugo, Y. Uchida, A. Cassidy, Sulfation of genistein alters its antioxidant properties and its effect on platelet aggregation and monocyte and endothelial function, Bioch. Biophys. Acta-General Subjects 1670 (2004) 229–237.
[38] W. Liu, Z.J. Song, N.C. Liang, J. She, L.E. Mo, Inhibitory effects of sodium quercetin monosulfate on pig platelet aggregation induced by thrombin, Zhongguo Yao Li Xue Bao 20 (1999) 623–626.
[39] W. Liu, N.C. Liang, Inhibitory effect of disodium qurecetin-7,4'-disulfate on aggregation of pig platelets induced by thrombin and its mechanism, Acta Pharmacologica Sinica 21 (2000) 737–741.
[40] Z. Wang, J. You, Y. Huang, et al., Effect of resveratrol on platelet aggregation in vivo and in vitro, Chin. Med. J. (Engl) 115 (2002) 378–380.
[41] E.F. Mammen, P.C. Comp, R. Gosselin, C. Greenberg, W.K. Hoots, C.M. Kessler, E.C. Larkin, D. Liles, D.J. Nugent, PFA-100 (TM) system: a new method for assessment of platelet dysfunction, Semin. Thromb. Hemost. 24 (1998) 195–202.
[42] O. Toth, A. Calatzis, S. Penz, H. Losonczy, W. Siess, Multiple electrode aggregometry: a new device to measure platelet aggregation in whole blood, Thromb. Haemost. 96 (2006) 781–788.
[43] A. Salat, S. Kroess, D. Felfernig-Boehm, M. Felfernig, T. Fleck, D. Schmidt, S. Pulaki, M.R. Mueller, Comparison of in vitro closure time (PFA-100) with

whole blood electrical aggregometry and platelet surface antigen expression in healthy volunteers, Thromb. Res. 105 (2002) 205–208.
[44] C. Jambor, C.F. Weber, K. Gerhard, W. Dietrich, M. Spannagl, B. Heindl, B. Zwissler, Whole blood multiple electrode aggregometry is a reliable point-of-care test of aspirin-induced platelet dysfunction, Anesth. Analg. 109 (2009) 25–31.
[45] A. Johnson, N. Dovlatova, S. Heptinstall, Multiple electrode aggregometry and P2Y(12) antagonists, Thromb. Haemost. 99 (2008) 1127–1129.
[46] K. Kottke-Marchant, J.B. Powers, L. Brooks, S. Kundu, D.J. Christie, State-of-the-art review: the effect of antiplatelet drugs, heparin, and preanalytical variables on platelet function detected by the Platelet Function Analyzer (PFA-100®), Clin. Appl. Thromb. Hemost. 5 (1999) 122–130.
[47] M. Homoncik, B. Jilma, N. Hergovich, P. Stohlawetz, S. Panzer, W. Speiser, Monitoring of aspirin (ASA) pharmacodynamics with the platelet function analyser PFA-100®, Thromb. Haemost. 83 (2000) 316–321.
[48] US 11/262,509 Dual Anti-Platelet and Anti-Coagulant Pyridoxine Analogues (filing date: October 28, 2005) EP 058501180: Dual Anti-Platelet and Anti-Coagulant Pyridoxine Analogues (filing date: October 28, 2005).
[49] P. Stefanic Anderluh, M. Anderluh, J. Ilas, J. Mravljak, M. Sollner Dolenc, M. Stegnar, D. Kikelj, Toward a novel class of antithrombotic compounds with dual function. Discovery of 1,4-benzoxazin-3(4H)-one derivatives possessing thrombin inhibitory and fibrinogen receptor antagonistic activities, J. Med. Chem. 48 (2005) 3110–3113.
[50] J. Ilas, T. Tomasic, D. Kikelj, Novel potent and selective thrombin inhibitors based on a central 1,4-benzoxazin-3(4H)-one scaffold, J. Med. Chem. 51 (2008) 2863–2867.
[51] M. de Candia, F. Liantonio, A. Carotti, R. De Cristofaro, C. Altomare, Fluorinated benzyloxyphenyl piperidine-4-carboxamides with dual function against thrombosis: inhibitors of factor Xa and platelet aggregation, J. Med. Chem. 52 (2009) 1018–1028.