Universidade de Lisboa

Faculdade de Farmácia

Interaction of Flavonoids and Derivatives with the Activity of Coagulation Enzymes *in vitro*

Marco Daniel Kandziouba Agostinho

Orientador: PharmDr. Alejandro Carazo, Assistant Professor, Faculty of Pharmacy in Hradec Králové, Charles University, Czech Republic.

Coorientadora: Professora Cristina Maria Leitão de Carvalho, Professora Associada, Faculdade de Farmácia da Universidade de Lisboa, Portugal.

Mestrado Integrado em Ciências Farmacêuticas

2022

Universidade de Lisboa

Faculdade de Farmácia

Interaction of Flavonoids and Derivatives with the Activity of Coagulation Enzymes *in vitro*

Marco Daniel Kandziouba Agostinho

Trabalho Final de Mestrado Integrado em Ciências Farmacêuticas apresentado à Universidade de Lisboa através da Faculdade de Farmácia

Orientador: PharmDr. Alejandro Carazo, Assistant Professor, Faculty of Pharmacy in Hradec Králové, Charles University, Czech Republic

Coorientadora: Professora Cristina Maria Leitão de Carvalho, Professora Associada, Faculdade de Farmácia da Universidade de Lisboa, Portugal

2022

Para Ivan Kandziouba e Alexandra Figueiredo.

Resumo

As doenças cardiovasculares têm uma das mais elevadas taxas de incidência e é uma das principais causas de morte no mundo. Entre os principais fatores de risco que levam ao desenvolvimento de doenças cardiovasculares estão os distúrbios de coagulação. Duas das enzimas de grande importância no processo de coagulação são a trombina e o fator X ativado (FXa). Um aumento da sua atividade pode muito facilmente levar a uma desregulação do processo normal de coagulação. A terapêutica convencional existente para o tratamento de doenças cardiovasculares e distúrbios de coagulação têm efeitos adversos, portanto surge um interesse crescente pelos benefícios do uso de substâncias de origem natural, como é o caso dos flavonoides.

Flavonoides são um grupo de substâncias polifenólicas de origem vegetal, presentes em frutas e vegetais, mas também em suplementos alimentares. São conhecidos por terem uma capacidade antioxidante (captação de radicais livres e quelação de metais), ação anti-inflamatória e por terem possíveis implicações benéficas na saúde e bem-estar humanos, como tratamento e prevenção de cancro, doença cardiovascular, entre outras patologias.

Neste estudo eu pretendi avaliar se e de que forma os flavonoides são capazes de interagir e influenciar a atividade da trombina e do FXa, ambos presentes no processo de coagulação. Foram utilizados 30 compostos no total, entre eles flavonoides e seus derivados, e estudada a sua interação com as enzimas selecionadas da cascata de coagulação. O método utilizado para o estudo foi o Ensaio de Substrato Cromogénico, que se baseia na medição da absorvância de p-nitroanilina libertada durante a reação entre a enzima em estudo e o substrato cromogénico utilizado. Foi observado que de entre todos os compostos apenas dois apresentavam uma ação inibitória direta na atividade da trombina (baicaleína e morina). Quanto ao Fxa todos os compostos, com exceção de três, apresentaram potencial de inibição da enzima de forma dose-dependente. Estes resultados demonstram que os flavonoides têm potencial para virem a ser usados como bases estruturais para o desenvolvimento de novos inibidores diretos do Fxa, que sejam seguros, oralmente biodisponíveis e de origem natural.

Palavras-chave: coagulação, flavonoides, trombina, Fxa, ensaio de substrato cromogénico.

Abstract

Cardiovascular diseases have one of the highest incidence rates and are one of the leading causes of death in the world. Among the main risk factors that lead to the development of cardiovascular diseases are clotting disorders. Two of the enzymes of great importance in the clotting process are thrombin and factor X activated (FXa). An increase in its activity can very easily lead to a disruption of the normal clotting process. The existing conventional therapy for the treatment of cardiovascular diseases and clotting disorders has adverse effects, so there is a growing interest in the benefits of using substances of natural origin, such as flavonoids.

Flavonoids are a group of polyphenolic substances of plant origin, present in fruits and vegetables, but also in food supplements. They are known for having antioxidant capacity (free radical scavenging and metal chelation), anti-inflammatory action and for having possible beneficial implications for human health and well-being, such as treatment and prevention of cancer, cardiovascular disease, among other pathologies.

In this study I intended to assess whether and how flavonoids are able to interact and influence the activity of thrombin and FXa, both present in the clotting process. Thirty compounds were used in total, among them flavonoids and some of their derivatives, and their interaction with the selected enzymes of the coagulation cascade, was studied. The method used for the study was the Chromogenic Substrate Assay, which is based on measuring the absorbance of p-nitroaniline released during the reaction between the enzyme under study and the chromogenic substrate used. It was observed that among all compounds, only two had a direct inhibitory action on thrombin activity (baicalein and morin). As for FXa, all compounds, with the exception of three, showed the potential to inhibit the enzyme in a dose-dependent manner. These results demonstrate that flavonoids have the potential to be used as structural bases for the development of new direct FXa inhibitors that would be safe orally bioavailable and of natural origin.

Keywords: coagulation, flavonoids, thrombin, FXa, chromogenic substrate assay.

Index

Figure Index

1 Abbreviations

ADP – adenosine diphosphate

apoB - apolipoprotein B

cAMP - cyclic adenosine monophosphate

CVD – cardiovascular disease

DMSO - dimethylsulfoxide

EDTA - ethylenediaminetetraacetic acid

FXa – factor X activated

Hsd - hesperidin

Hst – hesperetin

LDL - low-density lipoprotein

MCP-1 - monocyte chemoattractant protein-1

NO - nitric oxide

O-DMA - O-desmethylangolensin

PBS - phosphate-buffered saline

PI/PIP - phosphatidylinositol/phosphatidylinositol phosphate

PLA_2 - phospholipases A_2

PTK - protein-tyrosine kinases

SD – standard deviation

TBS - tris-buffered saline

TF – tissue factor

TNF-α - tumor necrosis factor-α

vWF - von Willebrand factor

2 Introduction

Flavonoids are polyphenolic compounds and can be found in medicinal plants, fruits and vegetables. Common dietary sources are strawberries, blueberries and apples (1,2), cocoa and chocolate (3), grapes, oranges, legumes, nuts, olive oil, tea, coffee (4) and wine. These compounds have important roles in the functioning of plants, such as reproduction, development and protection from predators and pathogens (such as bacteria, fungi, viruses and insects) (5). They are also responsible for providing attractiveness to the fruits, flowers and leaves of plants, through their flavor and color (6). Polyphenols have a strong antioxidant activity, which protects plants from the oxidant effect of ultraviolet (UV) radiation damage. They act as metal chelators as well. Other functions of flavonoids in plants include stimulating the colonization of nitrogen- fixing mycorrhizal fungi and bacteria, in order to form mycorrhizal symbioses beneficial for the plant (7), and also they can be produced to serve as an acclimation factor to environmental stressors (8–11). For this reason the flavonoid content in foods from different climatic conditions might differ based on the temperature, day length and solar radiation exposure (12). For example warm temperatures during growing of plants and exposure to UV-B light stimulates the accumulation of flavonoids (13).

There are more than 8000 recognized polyphenols, containing leastways 4000 flavonoids in plants. (5,14,15) The basal structure of flavonoids consists of 15 carbon atoms with two terminal aromatic rings linked together through an oxygenated heterocycle (C_6-C_3-C_6). Flavonoids can be divided in six main classes (flavonols, flavones, flavanols, flavanones, isoflavones and anthocyanidins), taking into account their molecular structures. They are represented in Figure 1 (16–18). **Flavonols** (i.e., myricetin, kaempferol, quercetin) are found in broccoli, tomatoes, celery, onions, kale, apples and grapes; **flavanones** (naringenin, hesperetin) are present in citrus fruits (oranges, lemons, grapefruits); **flavanols** (catechin, epicatechin) occur in green tea, red grapes and red wine; **isoflavones** (genistein) appear in soy and tofu; **flavones** (luteolin, apigenin) are present in hot peppers, celery, parsley and thyme; **anthocyanins** (cyanidin, malvidin, petunidin, peonidin) are responsible for the bright colors of strawberries, blueberries, bilberry, raspberries, elderberry, black currant, cherries, pomegranate, red onions and red wine(19).

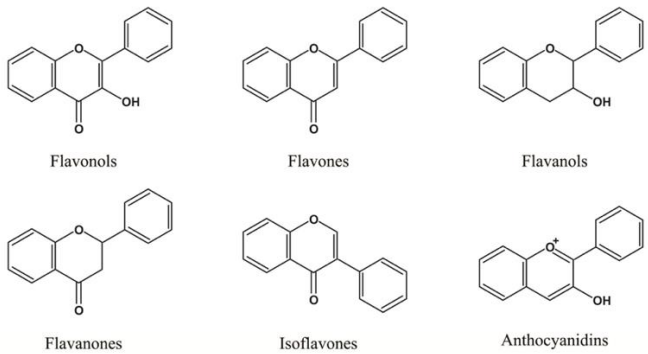


Figure 1. Chemical structures of the main classes of flavonoids. (20)

Although flavonoids are present in a wide variety of foods, there are some differences in the types and quantity of flavonoids consumed in different parts of the world. This happens because of differences in lifestyle, cultural food habits, gender and socioeconomic status of world

population, which together with environmental factors will contribute to differences in disease prevalence (21–23). Worldwide, the intake of dietary flavonoids is between 50 and 400 mg/day (24–27). The major sources of flavonoids in Westernized regions such as Australia, US and Europe are citrus fruits or vegetable juices, tea and wine, with an estimated intake ranging from 200 mg/day in Australia (28) to 500 mg/day in Europe, mostly because of the higher tea consumption (29). In Asian countries (China, Japan and Korea) the total daily intake of flavonoids ranges from 90 to 196 mg/day, but here the intake of isoflavones, ranging between 10 to 60 mg/day, is much higher than in Europe and the Americas where it is around 1-2 mg/day (24,26,27,30,31).

Flavonoids are known for their role in the prevention of coronary heart diseases. It is suggested by epidemiological studies that a diet rich in fruits and vegetables has a cardioprotective effect, being this beneficial effect mainly attributed to their flavonoid content (32,33). In fact, for the same reason Mediterranean countries have a lower mortality rate from cardiovascular diseases (CVD), in comparison with other territories (5). Other actions of flavonoids include anti-allergic, anti-inflammatory, antibacterial, antiviral, anti-thrombotic, anti-carcinogenic and antioxidant activities (34–36). Various epidemiological studies have shown that dietary flavonoids from different sources reduce the risk and mortality of coronary and cardiovascular heart disease (37–39). The protective effects of flavonoids against CVD complications might be associated with anti-platelet properties, among other mechanisms. It is has been reported that flavonoids can inhibit platelet adhesion, secretion and aggregation (40,41). Also they can inhibit different enzymes involved in cellular signaling (42), to exert anticoagulant activity (43), and to increase nitric oxide (NO) formation (41). During this COVID-19 pandemic, there is a growing scientific interest in the search for flavonoid compounds that can reduce COVID-19-infected cardiovascular malfunctioning by blocking the viral entry at the ACE2 receptor(44).

The aim of this project was to further understand how flavonoids interact with coagulation enzymes. To answer this, we must first understand how the process of hemostasis works. Hemostasis is the staunching of bleeding by the formation of a thrombus, and it happens whenever a blood vessel is injured. The first response of a vessel when damaged is constriction, but the most important processes are platelet aggregation and the formation of a platelet plug (primary hemostasis) and blood coagulation (secondary hemostasis).

The platelet plug is formed by the adhesion of platelets, via von Willebrand factor (vWF), to exposed subendothelial matrix from the vessel. This binding drives the platelets to secrete some chemical substances, such as adenosine diphosphate (ADP) and serotonin, that will induce changes in the structure of the platelets, which ultimately will lead to platelet aggregation, by the adhesion of new platelets to the previous ones, forming a platelet plug. Thromboxane A_2 also plays a key role in the platelet aggregation, by stimulating it. During the formation of the plug there is vasoconstriction as a result of platelet activity and the chemicals released (45). (Figure 2.)

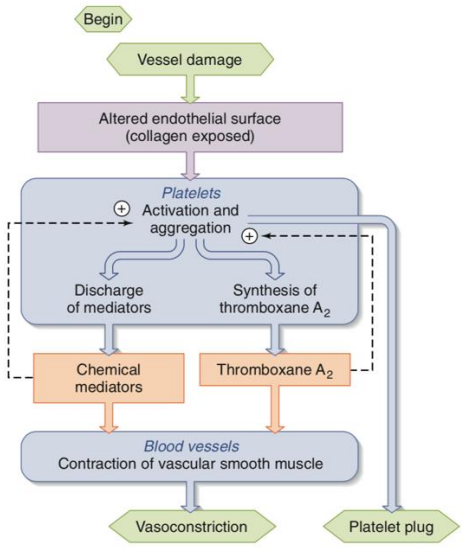


Figure 2. Events during the formation of a platelet plug.(46)

This is the primary mechanism to repair the damaged vessel wall. In the meantime, the process of blood coagulation and the formation of the clot begins. The clotting process starts with the contact between blood and the underlying tissue of the injured vessel. This will initiate a cascade of chemical activations. In every step of the cascade one plasma protein is activated and converted to a proteolytic enzyme, which in turn is going to catalyze the activation of the next enzyme in the sequence. After several of these activations, the plasma protein prothrombin is converted to thrombin. Thrombin is going to catalyze the reaction of splitting polypeptides from the large plasma protein fibrinogen, that will bind and form fibrin, the main protein polymer present in the clot. Fibrin is stabilized by factor XIIIa, which is formed from the activation of protein factor XIII by thrombin (46). (Figure 3.)

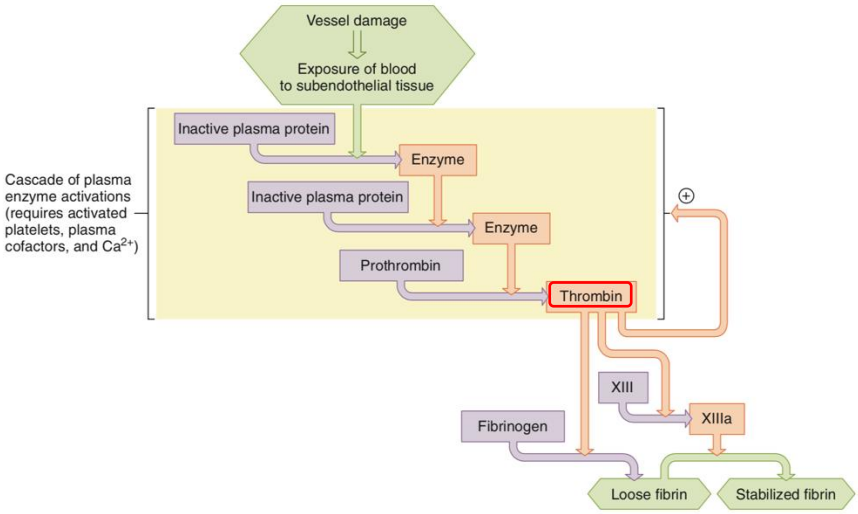


Figure 3. Clotting pathway simplified.(46)

As we can see, thrombin already has three important functions so far: catalyzes the formation of loose fibrin, activates factor XIII, but also has a positive feedback effect on its own formation and is important in stimulating the activation of platelets(46).

Now we will analyze in more detail the clotting cascade. There are two pathways that begin after the vessel damage and merge before the prothrombin-thrombin reaction. They both might

seem independent but have several points of interaction. The two pathways are the intrinsic pathway and the extrinsic pathway, and they can be better understood in the following figure 4.

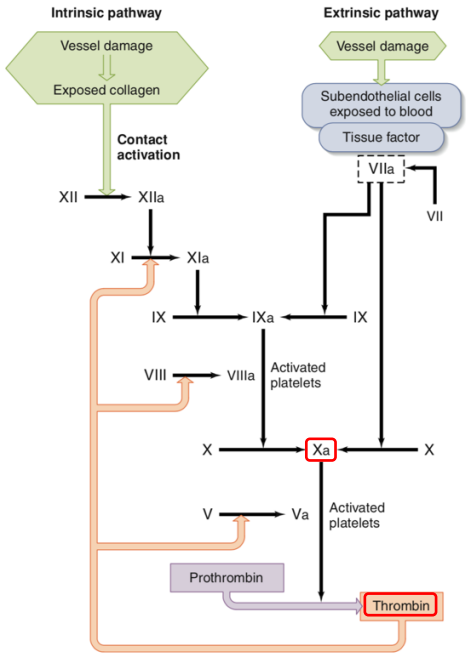


Figure 4. Intrinsic and Extrinsic clotting pathways.(46)

In the extrinsic pathway, tissue factor (TF) that was exposed from the extravascular tissues is going to form a complex with factor VII, thus activating it into FVIIa. The complex of TF and FVIIa will activate factor X and factor IX. Factor Xa (FXa) in the presence of its cofactor factor Va will activate prothrombin to generate thrombin (47). Thrombin, as already mentioned, is responsible for positive feedback activation of coagulation, and will act in the intrinsic pathway. It activates factor XI, which will activate factor IX, and thrombin also activates cofactors VIII and V (48).

Extrinsic pathway, via TF, is usually the initiating clotting way in the body, to the detriment factor XII beginning of the intrinsic pathway. Thus, thrombin is generated only by the extrinsic pathway. This will only produce a small initial amount of thrombin, however it will be enough to trigger the intrinsic pathway and enhance the prothrombin-thrombin step itself, and like this produce the large amounts of thrombin required for adequate coagulation (46).

This is why FXa is so important in the coagulation cascade, because alone it is responsible for the majority of the thrombin. **One molecule of FXa generates more than 1000 thrombin molecules** (49).

The existing conventional therapeutic options for thrombotic and coagulation disorders can have side effects and drug interactions. So, there has been a rise in interest for the search of natural based alternatives. In this paper I evaluate the effectiveness of flavonoid capacity to inhibit coagulation enzymes and thus serve as human antithrombotic and anticoagulation agents.

3 Objectives

The main goal of this work is to study how different flavonoids (flavones, flavonols, flavanols, flavanones, isoflavones, flavanonol) and some of their derivatives affect the activity of coagulation enzymes, namely thrombin and FXa, with the possibility to consider its benefits in therapeutic use, in the prevention and treatment of thrombotic and cardiovascular diseases, and to extrapolate to an *in vivo* reality. Also, to analyze how these flavonoids can influence, or maybe even substitute existing therapies with anticoagulants and antiplatelet agents.

4 Materials and Methods

4.1 Chemicals

Apigenin, baicalein, catechin, chrysin, daidzein, O-desmethylangolensin (O-DMA), diosmin, R,S-equol, 4-ethylphenol, flavone, 5-OH flavone, 7-OH flavone, genistein, hesperetin, hesperidin, isorhamnetin, kaempferol, luteolin, morin, mosloflavone, myricetin, naringenin, negletein, phloroglucinol, quercetin, resorcinol, rutin, tri-OH_2 rutin, silymarin and taxifolin were purchased from Sigma-Aldrich (Sigma-Aldrich, Sant-Louis, MO, USA).
Chromogenic substrates S-2238 and S-2222 were purchased from Chromogenix (Milan, Italy). Human plasma thrombin (Sigma-Aldrich; 605190-100U-M) and bovine plasma FXa (Sigma-Aldrich; F9302-50UG) were purchased from Sigma-Aldrich (Sigma-Aldrich, Sant-Louis, MO, USA). Dabigatran and rivaroxaban were purchased from Sigma-Aldrich (Sigma-Aldrich, Sant-Louis, MO, USA). Dimethylsulfoxide (DMSO) was purchased from Sigma-Aldrich (Sigma-Aldrich, Sant-Louis, MO, USA).

*4.2 Samples preparation **Thrombin Chromogenic assay***

The Tris-buffered saline (TBS) buffer was prepared using Tris (final concentration 50 mM) and NaCl (final concentration 150 mM), diluted in phosphate-buffered saline (PBS) and the pH adjusted to 7.4. Thrombin sample would be diluted in TBS to a concentration of 0.75 U/ml. Flavonoid compounds (luteolin, apigenin, chrysin, baicalein, myricetin, mosloflavone, flavone, 5-OH flavone, 7-OH flavone, diosmin, negletein, quercetin, kaempferol, tri-OH_2 rutin, rutin, isorhamnetin, morin, naringenin, hesperetin, hesperidin, taxifolin, daidzein, genistein, O-DMA, R,S-equol, catechin, silymarin, resorcinol, 4-ethylphenol and phloroglucinol), with an initial dilution in 50 % DMSO to 1 mM, were diluted in TBS to concentrations of 100 µM, 75 µM, 50 µM, 25 µM, 10 µM and 1 µM. DMSO (0,1% in TBS) was used as negative control and dabigatran (in TBS at concentrations of 10 µM, 1 µM and 0,1 µM) was used as positive control. Chromogenic substrate S-2238 was diluted in TBS to a concentration of 0,375 mM.

*4.3 Determination of activity of **Thrombin***

The activity of Thrombin was measured by the absorbance of p-nitroaniline produced by the reaction of thrombin and the chromogenic substrate S-2238 (H-D-Phe-Pip-Arg-pNA•2HCl) and the consequent hydrolysis. Absorbance measurements were performed using a 96-well microplate reader, model Hidex Sense Beta Plus (Hidex Oy, Turku, Finland). Firstly, 75 µL of each concentration of one test compound would be pipetted in the first column of wells, then the same with the other test compound in the second column, and 75 µL of DMSO in one well of the third column and 75 µL of dabigatran (in one of the chosen concentrations) on the next well. In each measurement two compounds would be used. Then it was added 75 µL of thrombin (0,75 U/ml) to all of the previously mentioned wells. After the addition of thrombin, the microplate would be put inside the microplate reader and start the incubation at 37ºC for 10 minutes. Following the incubation, the microplate would be taken out of the reader and 40 µL of chromogenic substrate S-2238 (0,375 mM) will be added to every well as quickly as possible.

The absorbance measurement was started at 405 nm at 37 ºC. The absorbance values were monitored every 1 min for 30 min. The results were presented as activity percent of control value according to compound concentration.

In the cases where the test compounds would have color, in order to take into account the influence that the color would have in the absorbance measurement, we would have one column of wells with one compound (as in the method explained previously) and another column with 75 µL of the same compound but with 115 µL of TBS buffer (where thrombin and S-2238 would not be added). This way the sample with compound plus buffer served as blank. Afterwards during the result treatment, the absorbance result of the blank would be subtracted from the measurement of the regular sample (compound+thrombin+S-2238) to obtain the corrected values of absorbance, without the influence of the color of the compound sample.

*4.4 Samples preparation **FXa Chromogenic assay***

The Tris-HCl buffer was prepared using Tris-HCl (final concentration 50 mM), ethylenediaminetetraacetic acid (EDTA) (final concentration 7.5 mM) and NaCl (final concentration 150 mM) diluted in dH_2O and the pH adjusted to 7.4. FXa sample would be diluted in Tris-HCl to a concentration of 2 U/ml. Flavonoid compounds (luteolin, apigenin, chrysin, baicalein, myricetin, mosloflavone, flavone, 5-OH flavone, 7-OH flavone, diosmin, negletein, quercetin, kaempferol, tri-OH_2 rutin, rutin, isorhamnetin, morin, naringenin, hesperetin, hesperidin, taxifolin, daidzein, genistein, O-DMA, R,S-equol, catechin, silymarin, resorcinol, 4-ethylphenol and phloroglucinol), with an initial dilution in 50 % DMSO to 1 mM, were diluted in Tris-HCl to concentrations of 100 µM, 75 µM, 50 µM, 25 µM, 10 µM and 1 µM. DMSO (0,1% in Tris-HCl) was used as negative control and rivaroxaban (in Tris-HCl at concentrations of 5 µM, 1 µM and 0,1 µM) was used as positive control. Chromogenic substrate S-2222 was diluted in Tris-HCl to a concentration of 0.8 mM.

*4.5 Determination of activity of **FXa***

The activity of FXa was measured by the absorbance of p-nitroaniline produced by the reaction of FXa and the chromogenic substrate S-2222 (Bz-Ile-Glu(g-OR)-Gly-Arg-pNA·HCl) and the consequent hydrolysis. Absorbance measurements were performed using a 96-well microplate reader, model Hidex Sense Beta Plus (Hidex Oy, Turku, Finland). Firstly, 75 µL of each concentration of one test compound would be pipetted in the first column of wells, then the same with the other test compound in the second column, and 75 µL of DMSO in one well of the third column and 75 µL of rivaroxaban (in one of the chosen concentrations) on the next well. In each measurement two compounds would be used. Then it was added 75 µL of FXa (2 U/ml) to all of the previously mentioned wells. After the addition of FXa, the microplate would be put inside the microplate reader and start the incubation at 37ºC for 1 minute. Following the incubation, the microplate would be taken out of the reader and 50 µL of chromogenic substrate S-2222 (0.8 mM) will be added to every well as quickly as possible. The absorbance measurement was started at 405 nm at 37 ºC. The absorbance values were monitored every 1 min for 15 min. The results were presented as activity percent of control value according to compound concentration.

In the cases where the test compounds would have color, in order to take into account the influence that the color would have in the absorbance measurement, we would have one column of wells with one compound (as in the method explained previously) and another column with 75 µL of the same compound but with 125 µL of Tris-HCl buffer (where FXa and S-2222 would not be added). This way the sample with compound plus buffer served as blank. Afterwards during the result treatment, the absorbance result of the blank would be subtracted from the measurement of the regular sample (compound+FXa+S-2222) to obtain the corrected values of absorbance, without the influence of the color of the compound sample.

4.6 Statistical analysis

Results are always presented as means ±SD of at least **two** independent experiments.

5 Results and Discussion

Firstly, an initial screening of all the results of the tested compounds at a concentration of **100 µM** on the activity of thrombin (Figure 5) and of FXa (Figure 6) using Chromogenic substrate assay were performed.

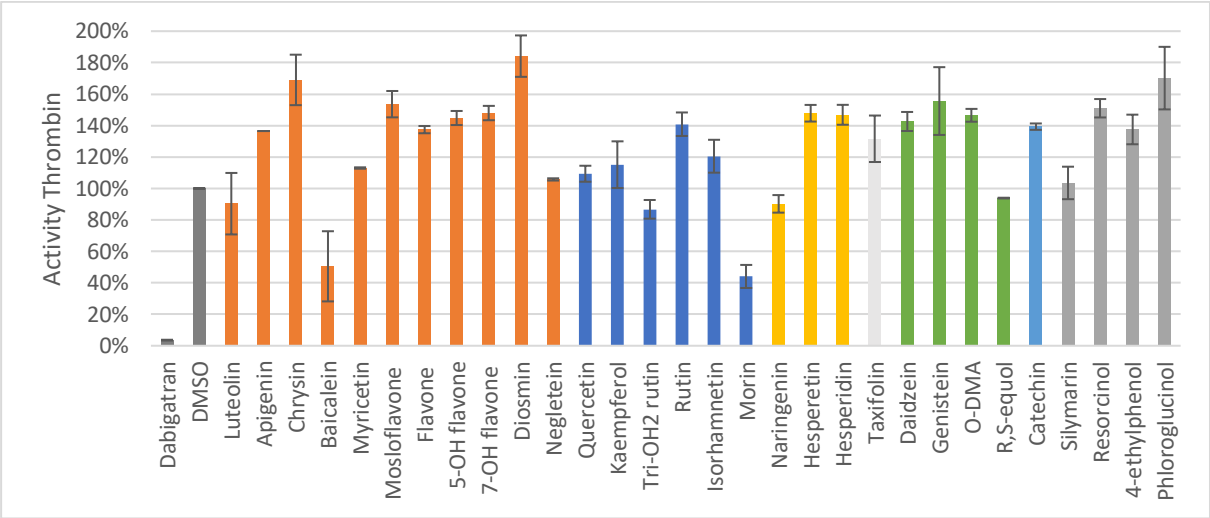


Figure 5. Effect of Flavonoid and derivative compounds on the activity of Thrombin. Results are presented as means with ±SD, in concentrations of 100 µM and results compared to DMSO 0,1%. Dabigatran 10 µM was used as positive control. The different colors represent the division of the compounds by their classes of flavonoids, being: dark grey – **controls**; orange – **flavones**; dark blue – **flavonols**; yellow – **flavanones**; light grey – **flavanonol**; green – **isoflavones**; light blue – **flavanols**; grey – **derivatives**.

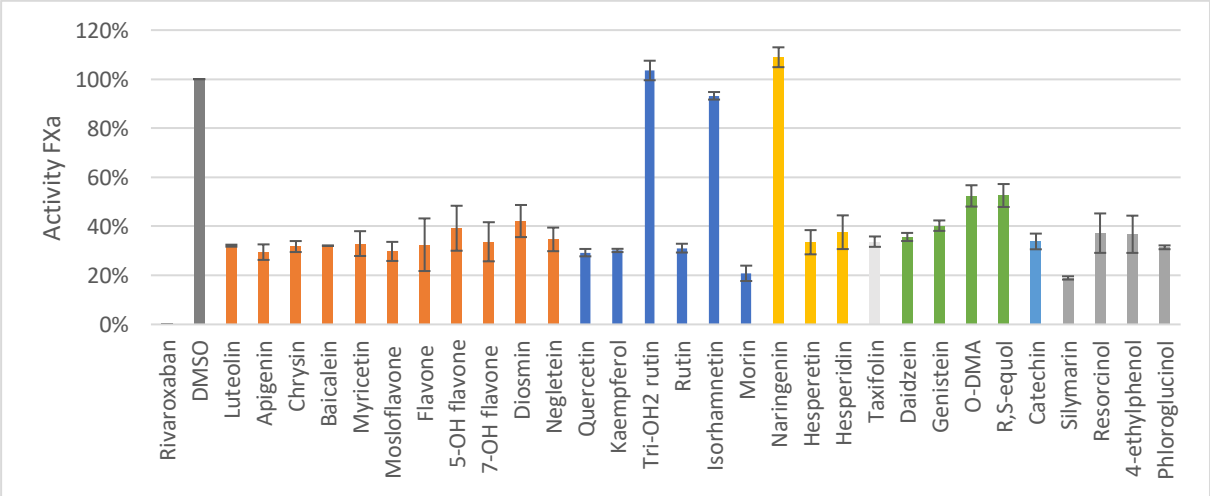


Figure 6. Effect of Flavonoid and derivative compounds on the activity of FXa. Results are presented as means with ±SD, in concentrations of 100 µM and compared to DMSO 0,1%. Rivaroxaban 5 µM was used as positive control. The different colors represent the division of the compounds by their classes of flavonoids, being: dark grey – **controls**; orange – **flavones**; dark blue – **flavonols**; yellow – **flavanones**; light grey – **flavanonol**; green – **isoflavones**; light blue – **flavanols**; grey – **derivatives**.

As we can see in both Figures 5 and 6, the value of DMSO, which is the negative control, is equal to 100%. So from now on, these are considered the normal activity levels of both thrombin and FXa.

By analyzing Figure 5 it is visible that the majority of compounds exert closely no direct influence on the activity of thrombin or even raise it. Two major exceptions to this trend are baicalein and morin, that are worth analyzing further in Figure 7, given the aim of studying the

anticoagulant and antithrombotic effects of this compounds. It should be mentioned that this results regarding thrombin are not in agreement with most known literature, because flavonoids tend to have an inhibitory effect on thrombin. More is explained on this matter further in the General Discussion segment.

5.1 Activity of Thrombin

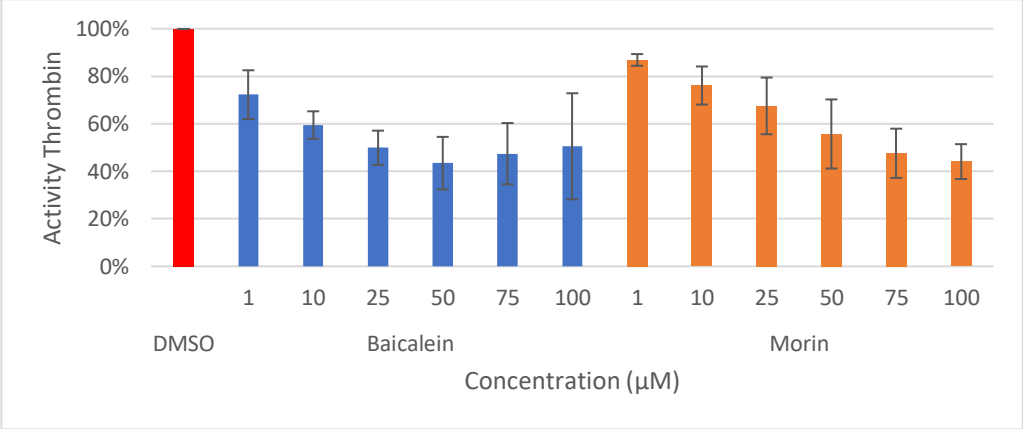


Figure 7. Effect of baicalein and morin on the activity of Thrombin. Results are presented as means with ±SD, in concentrations of 100 µM, 75 µM, 50 µM, 25 µM, 10 µM and 1 µM of both baicalein and morin. In red is represented DMSO.

In morin it is noticeable that the higher the concentration the greater its inhibiting power (Figure 7), making this very promising results. It also has good results in regard to the effect on FXa, that will be shown further. In baicalein there is also a similar trend, but the results are not so reliable given the high value of SD and the lack of linearity of the activity given the concentrations.

Regarding morin, it has been shown in previous work that morin has the ability to inhibit platelet-mediated thrombosis and also inhibits clot retraction (50). Clot retraction is an important coagulation event to take into account, because it involves the stiffness of the clot, making it denser and more resistant to fibrinolytic and thrombolytic therapy, and by that enhancing the risks of thromboembolism and other thrombotic diseases. As further stated by Nam et al., morin had significantly inhibited collagen-induced platelet aggregation, but also on thrombin-induced aggregation, although the effects were weaker. As well it managed to inhibit thrombin-induced clot retraction, and had effects on various substances that contribute to platelet activation and aggregation, such as the increase of Cyclic Adenosine Monophosphate (cAMP) production, suppressing TXA_2 production and granule secretion, among others (50). This suggests that morin might be interesting to further investigate in regard to safety and usefulness of its consumption by thrombotic patients.

5.2 Activity of FXa

Most of the studied compounds have an inhibitory effect on FXa, as seen in Figure 6, with exceptions of tri-hydrate rutin, isorhamnetin and naringenin, that show barely any influence on FXa. From now on we will take a closer look on the interaction of the compounds, dividing them by their flavonoid classes, starting with Figure 8 with the flavones. In Figure 9 are the molecular structures of the flavones tested.

5.2.1 Flavones

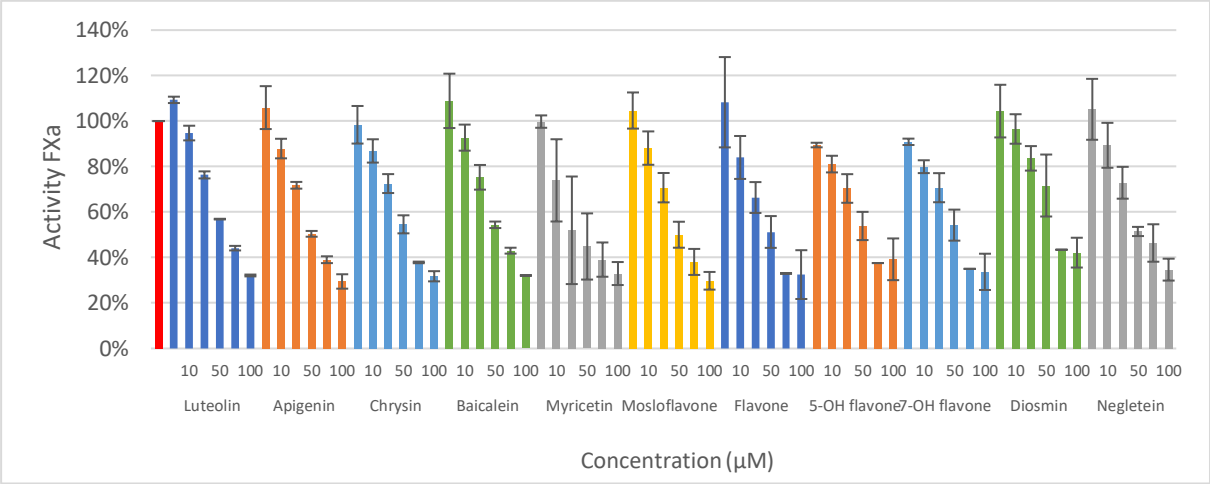


Figure 8. Effect of flavones on the activity of FXa. Results are presented as means with ±SD, in concentrations of 100 µM, 75 µM, 50 µM, 25 µM, 10 µM and 1 µM for all the flavones tested (luteolin, apigenin, chrysin, baicalein, myricetin, mosloflavone, flavone, 5-OH flavone, 7-OH flavone, diosmin and negletein). In red is represented DMSO.

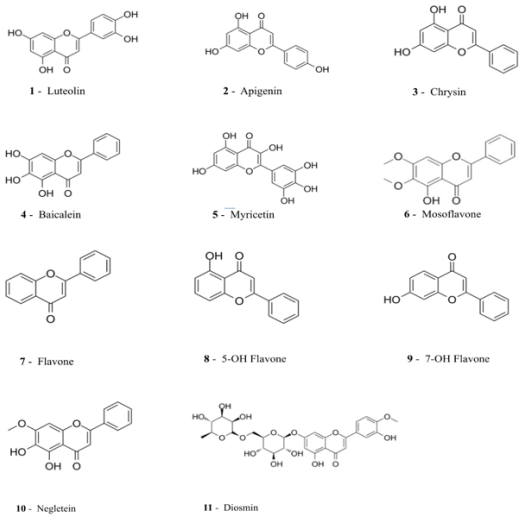


Figure 9. Molecular structure of tested flavones.

Figure 8 shows that all the tested compounds that belong to the flavones group have an inhibitory effect on FXa, in a dose-dependent way.

Flavones in general have several biological activities in humans. They elevate blood antioxidant enzyme activities, show an effect on biomarkers for heart disease and stroke, have a slight influence on platelet aggregation, decrease total cholesterol levels, increase venous tone and counter inflammatory mediators, demonstrating the anti-inflammatory activity of flavones. But flavones are not well absorbed, however this can be countered through processing and different forms of administration of flavones (51). Now we shall analyze more closely some of them.

Regarding luteolin, Choi et al. demonstrated that it directly inhibits the enzymatic activity of both thrombin and FXa, probably by binding to their active site. Their results also indicated that luteolin could have anticoagulant properties, by mediating the anticoagulation effect of blood coagulation factors, as well as identified an *in vitro* and *in vivo* potential antithrombotic action against fibrin clot formation (52).

Diosmin has poor water solubility that limits its applications in clinical use, however some strategies are developed to counter this. Complexation, preparation of diosmin nanoparticles or its use in micronized purified flavonoid fraction (as the one used in Daflon®) are some, which exhibit an increase in bioavailability, solubility, anti-oxidant and anti-inflammatory activities (53). Diosmin has various pharmacological activities, including antioxidant, anti-inflammatory, anti-diabetes and cardioprotective, among others. Previous papers show the anti-thrombotic and anti-hypertensive effects of diosmin (54,55). It is also useful for treating chronic venous insufficiency and varicose veins via the improvement of micro-circulation and attenuation of angiogenesis and inflammation (56).

Singh et al. tested the anti-inflammatory and immunomodulatory effects of mosloflavone and negletein. They showed a dose-dependent immunosuppressive response, inhibited the production of TNF-α and IL-1β in lymphocytes and inhibited the production of NO in macrophage J-774A cell line (57).

Myricetin has a wide range of pharmacological activities. Among them it has a strong anti-inflammatory activity, anti-tumor effect, cerebrovascular, anti-neurodegenerative and cardiovascular protective effects. Regarding its cardiovascular effects, myricetin reduces the level of oxidative stress and inflammatory cytokines by regulating IκB/NF-κB and Nrf2/HO-1 pathways in myocardial injury(58). Improves atherosclerosis by inhibiting CD36, thus regulating the uptake of cholesterol in macrophages(59). Inhibits angiotensin converting enzyme activity, and like this regulates vasodilation. The number and position of myricetin hydroxyl groups is relevant in this activity(60). Myricetin also functions as a protein disulfide isomerase and ERp5 reductase inhibitor, thereby mediating platelet aggregation and consequently thrombosis(61). In addition myricetin inhibits arachidonic acid induced platelet aggregation (62).

To my knowledge, no results were published on the direct influence of flavones on FXa, but it is known that there are benefits in their use and they should be investigated further. Flavones could be considered as new treatments in coagulation disorders and platelet aggregation and thrombotic disorders.

5.2.2 Flavonols

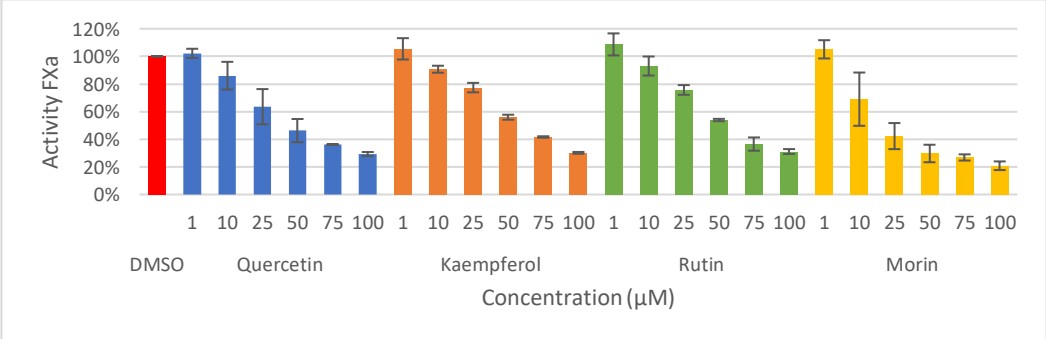


Figure 10. Effect of flavonols on the activity of FXa. Results are presented as means with ±SD, in concentrations of 100 µM, 75 µM, 50 µM, 25 µM, 10 µM and 1 µM for all the flavonols tested that showed inhibition of FXa (quercetin, kaempferol, rutin and morin). In red is represented DMSO.

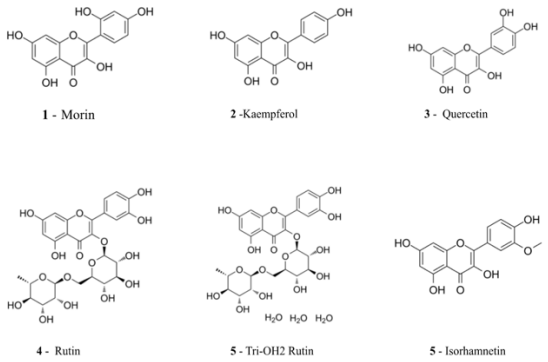


Figure 11. Molecular structure of tested flavonols.

As it is shown in Figure 10, all of this four flavonols have a dose-dependent inhibition on FXa. From a concentration of at least 50 µM there is a minimum restriction in FXa activity by 44%, this fact being more remarkable in Morin, where the activity is under 30%.

According to structures observations, flavonoids that have OH substituents at the R1 and R2 in the B ring (like quercetin in this case), they will inhibit FXa amidolytic activity. According to Bijak et al., quercetins A ring mimics arginine residue (P1) and forms reversible hydrogen bonds and a salt bridge with Asp189 in the S1 pocket of FXa, while the B ring interacts with S2 pocket. This means that quercetin will compete with the substrate for the enzyme active site, not allowing it to bind to FXa active center, and thus inhibiting its action (63).

Choi et al. demonstrated the inhibitory effects of kaempferol on both thrombin and FXa, and also on the formation of fibrin polymer. Delayed aggregation time of platelets, and exerted protection against thrombosis development in animal models. Kaempferol showed antithrombotic action and the potential to be a valuable agent against cardiovascular diseases(64).

Regarding morin, as mentioned before, it has several interesting activities, mainly in platelet activation and aggregation and clot retraction. Nevertheless, no data is available on previous work at analyzing its interaction with FXa, so proving the veracity of these results is important and this should be studied further in order to consider morin as a potential FXa inhibitor. Given the structural similarities between morin and quercetin and kaempferol this is very probable.

Rutin has effects in different conditions such as antioxidant, anti-inflammatory, antihypertensive and antithrombogenic effect. The mechanism for the last two is, respectively, is by action in the NO/guanylate cyclase pathway and inhibition of acetylcholinesterase (65), and scavenging of free radicals and lipid peroxides produced by activated platelets and inhibition of platelet aggregation (66).

5.2.3 Flavanones

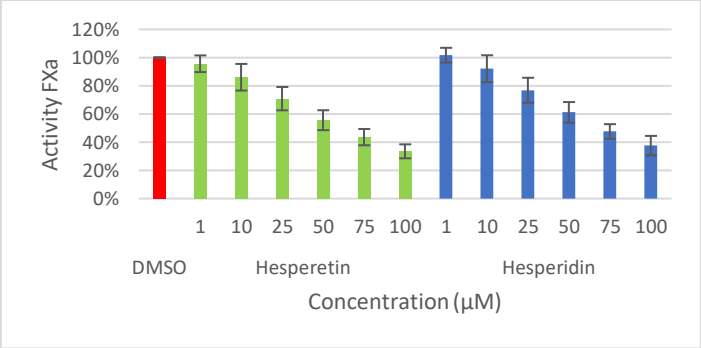


Figure 12. Effect of flavanones on the activity of FXa. Results are presented as means with ±SD, in concentrations of 100 µM, 75 µM, 50 µM, 25 µM, 10 µM and 1 µM for all the flavanones tested that showed inhibition of FXa (hesperetin and hesperidin). In red is represented DMSO.

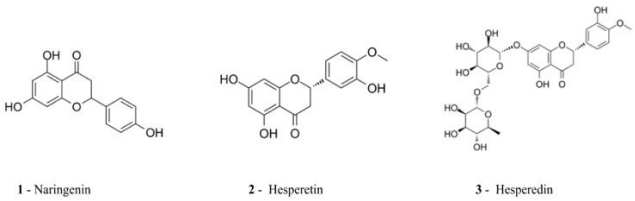


Figure 13. Molecular structure of tested flavanones.

In Figure 12 we can see that both hesperidin (Hsd) and hesperetin (Hst) have a dose-dependent inhibition on FXa.

It has been demonstrated that Hst has the ability to inhibit PLC-γ2 phosphorylation and cyclooxygenase-1 and thus inhibiting collagen and arachidonic acid induced platelet aggregation (67). Similarly, Hsd can inhibit ADP-, arachidonic acid-, collagen- and thrombin-induced rat platelet aggregation dose dependently, in vitro(68). Hsd and Hst inhibition of the gene expression of thromboxane A2 synthase and thromboxane B2 synthase of vascular endothelium, respectively, might also mediate their effects on coagulation (69). Yu et al. studied the influence of orally administered Hsd in comparison to aspirin on mouse tail bleeding time. The result was that Hsd increased the bleeding time more than aspirin (68). Furthermore, Hsd is one of the main components of Daflon® 500 mg (together with diosmin), that is demonstrated to significantly inhibit platelet functions, in vivo (70).

To my knowledge, no results were published on the direct effects of this compounds on FXa but given their already known effects on coagulation it wouldn't be surprising that they can also influence FXa. But nevertheless, further investigation is necessary in order to corroborate this hypothesis and to understand how this happens.

5.2.4 Flavanonol (Taxifolin)

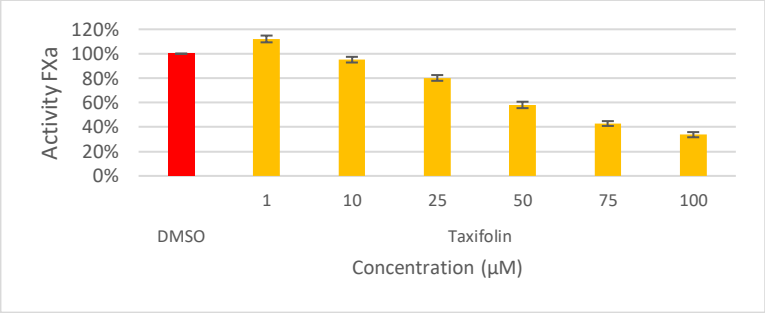
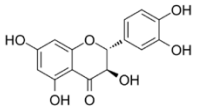


Figure 14. Effect of flavononol (taxifolin) on the activity of FXa. Results are presented as means with ±SD, in concentrations of 100 µM, 75 µM, 50 µM, 25 µM, 10 µM and 1 µM of taxifolin. In red is represented DMSO.



1 - Taxifolin

Figure 15. Molecular structure of taxifolin.

Taxifolin has a dose-dependent inhibitive action on FXa. It was demonstrated in previous experiments that taxifolin has, among other beneficial characteristics, the ability to dilate blood vessels, increase cerebral blood flow, improve microcirculation and inhibit platelet aggregation (71). Although, its clinical use in solid oral dosage form is limited because of poor solubility in water, its instability especially under alkaline conditions, and the fact that it is not absorbed in the intestine and is susceptible to intestinal microflora. Thus it would be necessary a formulation to enhance taxifolin release and residence time while in the stomach, where it can be easily absorbed (72).

No data on the interaction with taxifolin with FXa have been published, to the best of my knowledge, so further investigation would be necessary to evaluate the feasibility of its use as a FXa inhibitor.

5.2.5 Isoflavones

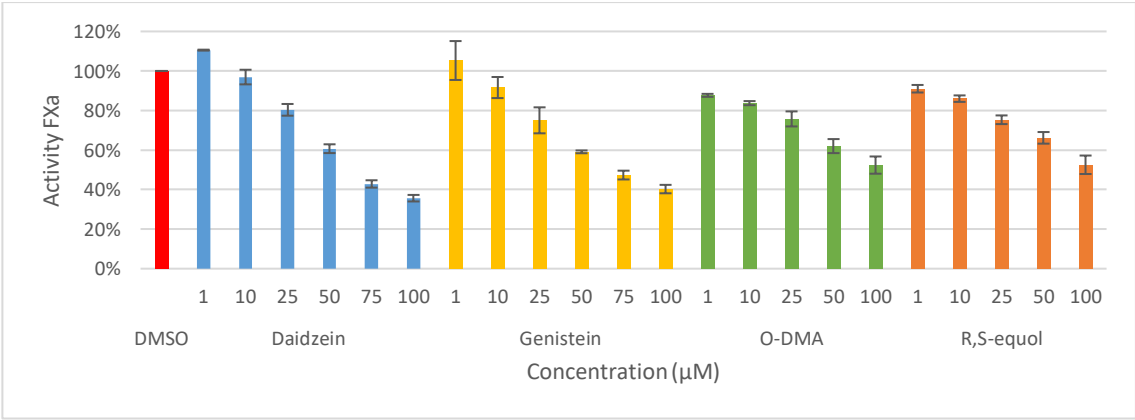


Figure 16. Effect of isoflavones on the activity of FXa. Results are presented as means with ±SD, in concentrations of 100 µM, 75 µM, 50 µM, 25 µM, 10 µM and 1 µM for all the isoflavones tested (daidzein, genistein). O-DMA and R,S-equol do not have a measuring at 75 µM concentration. In red is represented DMSO.

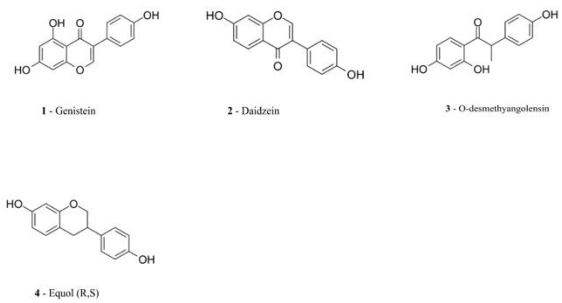


Figure 17. Molecular structure of tested isoflavones.

In Figure 16 we can see that all the compounds have a dose-dependent inhibitory effect on FXa, although higher concentrations of daidzein and genistein cause a slightly lower activity of FXa in comparison to the other two compounds. On the other hand, O-desmethylangolensin and R,S-equol (which are both metabolites of daidzein) show a much lower variation (roughly 40%) of influence on the activity of FXa depending on the concentration.

Soy-derived phytoestrogens (such as genistein and daidzein) have demonstrated anti-atherogenic effects on several levels among them reduction of oxidation of low-density lipoprotein (LDL), improves arterial compliance, reduced platelet activation and aggregation and inhibition of muscle cell proliferation (73).

Genistein is a compound that is proven to have effects on several disorders and diseases. Much of its effects are due to its capability to inhibit protein-tyrosine kinases (PTK) and phosphatidylinositol/phosphatidylinositol phosphate (PI/PIP) kinases, which are important for signal transduction pathways. It acts as an immunosuppressor agent by inhibiting lymphocyte activity, stabilizes mast cells and attenuates platelet aggregation. Also has a significant effect on inhibiting the proliferation of cancerous cells. Reduces apolipoprotein B (apoB) production and together with daidzein its secretion, thereby becoming a new possibility for treatment of cholesterol related defects. Genistein reduces the enhanced macrophage and serum levels of tumor necrosis factor-α (TNF-α), by directly inhibiting this inflammatory cytokine. Also it leads to an increase in NO production in human primary vascular endothelial cells (74). Many of these characteristics are common to daidzein as well.

Isoflavones have anti-aggregatory activity in human platelets *in vitro*, which might be enhanced by their antioxidant and radical scavenger characters (possible because of their hydroxyl groups in the structures). They appear to suppress monocyte chemoattractant protein-1 (MCP-1) secretion by human endothelial cell, that is involved in the recruitment of monocytes and T-cells into the arterial wall, playing a potential key role in atherogenesis.
This indicates that there are several potential mechanisms for the cardioprotective effect of isoflavones (75).

Although, to the best of my knowledge there are no published results on the direct influence of isoflavones on FXa, if these results could be confirmed by further investigation, it would be another proof of the cardioprotective characteristics of isoflavones, and that a relatively low exposure to isoflavones may contribute to a reduction in cardiovascular risk.

5.2.6 Flavanol (Catechin)

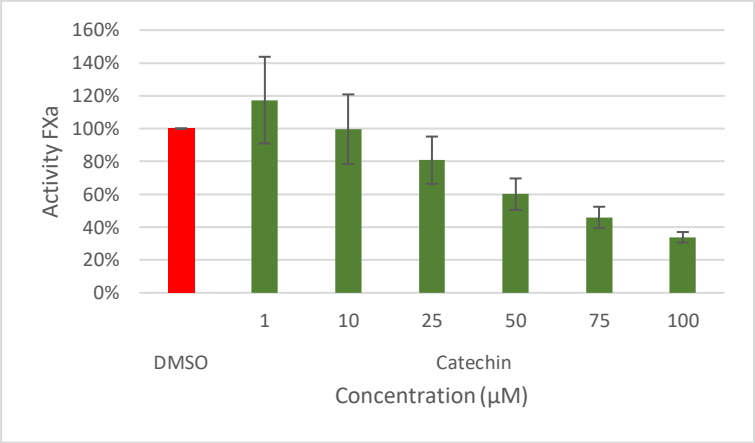
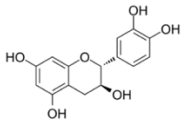


Figure 18. Effect of flavanol on the activity of FXa. Results are presented as means with ±SD, in concentrations of 100 µM, 75 µM, 50 µM, 25 µM, 10 µM and 1 µM for catechin. In red is represented DMSO.



1 - Catechin

Figure 19. Molecular structure of catechin.

In Figure 18 it is visible that catechin has a dose-dependent inhibitory effect on FXa.

Cesar et al. paper studied the inhibitory actions of catechin and epicatechin (not studied here) on toxins in snake venoms, mostly associated with changes in hemostasis. They demonstrated that catechin increase the clotting time, inhibits the action of phospholipases A_2 (PLA_2), is able to reduce hemolysis, inhibit fibrinogenolytic activity and thrombus dissolution (76). These inhibitions in protease-induced coagulant effects prevent the breakdown of coagulation cascade factors (77). There are some possible mechanisms to explain this inhibition of enzymatic toxins. One is the formation of complexes via hydrogen bonds through the aromatic and phenolic nuclei of polyphenols in general, and it also applies to catechin. A second mechanism might be the chelation of catechin with metal ions, important cofactors of enzymatic toxins. The structural regions involved in the chelation are between the 5-OH positions and the 4-oxo group, or between the 3´- and 4´-OH hydroxyls of the compounds(78). Another hypothesis considers the interactions between catechin and the amino acids present in the catalytic site of the enzymatic toxins, or in our case of FXa, that would result in a decrease in the activities. It would be necessary the presence of at least one hydroxyl group in order to occur a binding of catechin to the active site of the enzyme in favorable way (76).

5.2.7 Derivatives

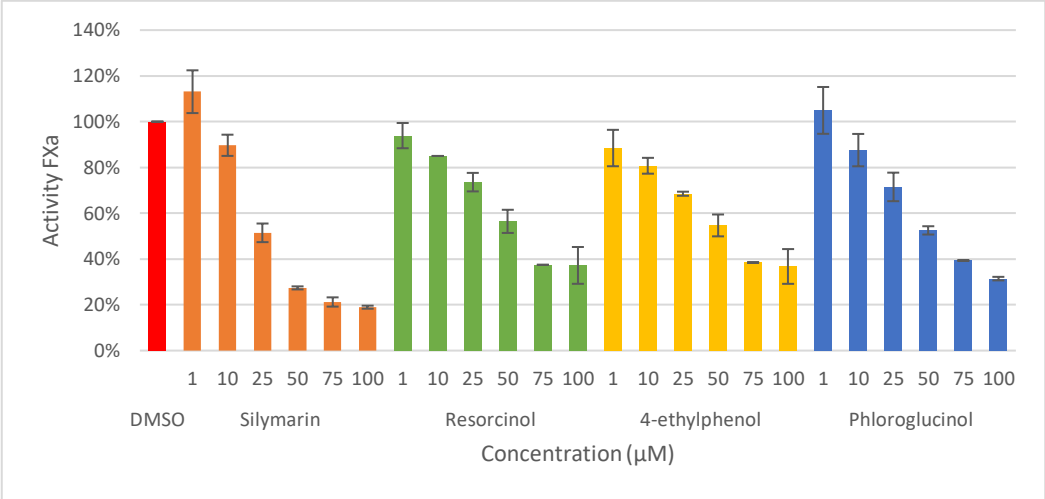


Figure 20. Effect of derivatives on the activity of FXa. Results are presented as means with ±SD, in concentrations of 100 µM, 75 µM, 50 µM, 25 µM, 10 µM and 1 µM for all the derivatives tested (silymarin, resorcinol, 4-ethylphenol and phloroglucinol). In red is represented DMSO.

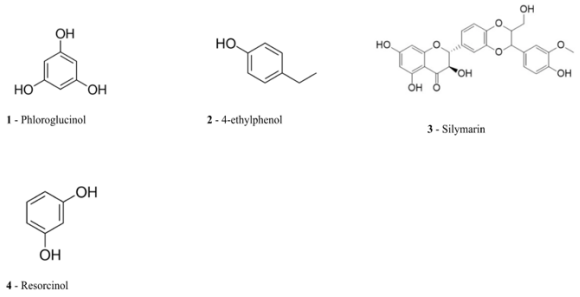


Figure 21. Molecular structure of tested derivatives.

In Figure 20 we can see that all of the derivatives exert an inhibition on FXa in a dose-dependent manner. Been the higher concentrations of silymarin the ones that have the most inhibition on FXa (more than 70%).

Silymarin is a component of the fruit extract of *Silybum marianum* (milk thistle), and is an isomeric mixture of flavonolignans, that are formed by combination of flavonoid and lignan structures. Silybin is the major bioactive component of silymarin (79). Silybin has several useful properties such as hepatoprotection, antioxidant, modulation of various cell-signaling pathways which leads to reduction of pro-inflammatory mediators(80). Previous studies also demonstrate that silybin is a potential anticancer and chemo-preventive agent, is able to inhibit serine proteases involved in the blood coagulation process and to reduce platelet response to agonists(79). Through observing the structure of flavonoids, the ones who inhibit FXa amidolytic activity have OH substituents at the positions R1 and R2 in B ring. In this case silybin has a hydroxyl in R2 and a methoxy group in R1 position (which might also have some interaction). This hydroxyl group of the B ring will bind with S4 pocket of FXa. This interaction will prevent the substrate from binding to FXa active center, meaning that silybin mimic the structure of the substrate and reversibly binds to FXa, thus competing for the active site (63). So, this result is consistent with previously done work.

Resorcinol is commonly available as topical applications due to its antibacterial, antioxidant and keratolytic actions. No data on the interaction with FXa or any coagulation enzymes.

Phloroglucinol is a polyphenolic compound with structure composed of an aromatic phenyl ring with three hydroxyl groups, which shows antimicrobial activity, among others. No data on the interaction with FXa or any coagulation enzymes.

5.2.8 General Discussion

In this paper we can see that the values of activity of thrombin were not following the trend of many other papers on the subject, that show a direct inhibitory effect of most flavonoids on thrombin. This might have happened due to procedures errors on my part or defects on the reagents used. Another more plausible hypothesis is that the results shown are experimental artifacts, meaning that they do not necessarily demonstrate the correct relationship between the experimental compounds and thrombin. Nevertheless, the results on the activity of FXa are of great interest, mainly because most of them haven´t been demonstrated in published work, and also because the influence on FXa will consequently exert an influence on thrombin.

The activated factor X forms prothrombinase complex on phosphatidylserine containing surface which is responsible for conversion of prothrombin to thrombin. One molecule of FXa generates more than 1000 thrombin molecules(49).

Anticoagulant treatment based only on FXa inhibition gives comparable therapeutic results as is the case of inhibition of thrombin. This knowledge has contributed to the search for new substances which could directly inhibit FXa activity (81).

6 Conclusion

Despite the broad pharmacological properties of flavonoids, research on the therapeutic efficacy, bioavailability issues and biotransformation of flavonoids is still missing, and it is needed to answer many questions. The bioactivity of flavonoids depends on their pharmacokinetic, metabolic and pharmacodynamic profile in the human body. It is necessary to evaluate the amount of flavonoids that need to be ingested in order to have an enough concentration in the human body to exert therapeutical action.

Flavonoids have the potential to serve as bases for the design of new nature-based, safe, orally bioavailable direct thrombin and FXa inhibitors. But they can also interact with several other plasma proteins involved in the coagulation cascade, so in order to achieve highly selective inhibitors, pharmacophores of the native structures could be used to design modified compounds.

To further understand how these flavonoids could be used, competitive inhibition assays between flavonoids and thrombin and FXa active centers show be done in order to determine the ability of this compounds to connect structurally to the enzymes.

These results demonstrate the capability of flavonoid compounds to inhibit coagulation enzymes, especially FXa, making them suitable candidates for further investigation and possible therapeutic use in the near future.

In conclusion, flavonoids have a vast array of disorders and diseases on which they can act, but there is still a way to go to fully understand the added value that they can provide in order for us humans to take another step towards a healthier and better life.

7 References

1. Miller K, Feucht W, Schmid M. Bioactive compounds of strawberry and blueberry and their potential health effects based on human intervention studies: A brief overview. Nutrients. 2019;11(7):1–12.
2. Schell J, Betts NM, Lyons TJ, Basu A. Raspberries improve postprandial glucose and acute and chronic inflammation in adults with type 2 diabetes. Ann Nutr Metab. 2019;74(2):165–74.
3. Marika M, Scoditti E, Carluccio MA, Kaltsatou A, Cicchella A. Effect of cocoa products and its polyphenolic constituents on exercise performance and exercise-induced muscle damage and inflammation: A review of clinical trials. Nutrients. 2019;11(7):1–15.
4. Farzaei MH, Bahramsoltani R, Abbasabadi Z, Braidy N, Nabavi SM. Role of green tea catechins in prevention of age-related cognitive decline: Pharmacological targets and clinical perspective. J Cell Physiol. 2019;234(3):2447–59.
5. Bravo L. Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance. Nutr Rev. 1998;56(11):317–33.
6. Timberlake CF, Henry BS. Plant pigments as natural food colours. Endeavour. 1986;10(1):31–6.
7. Taylor LP, Grotewold E. Flavonoids as developmental regulators. Curr Opin Plant Biol. 2005;8(3 SPEC. ISS.):317–23.
8. Grotewold E. The genetics and biochemistry of floral pigments. Annu Rev Plant Biol. 2006;57:761–80.
9. Albert NW, Lewis DH, Zhang H, Schwinn KE, Jameson PE, Davies KM. Members of an R2R3-MYB transcription factor family in Petunia are developmentally and environmentally regulated to control complex floral and vegetative pigmentation patterning. Plant J. 2011;65(5):771–84.
10. Anderson ER, Lovin ME, Richter SJ, Lacey EP. Multiple Plantago species (Plantaginaceae) modify floral reflectance and color in response to thermal change. Am J Bot. 2013;100(12):2485–93.
11. Hectors K, Van Oevelen S, Geuns J, Guisez Y, Jansen MAK, Prinsen E. Dynamic changes in plant secondary metabolites during UV acclimation in Arabidopsis thaliana. Physiol Plant. 2014;152(2):219–30.
12. Jaakola L, Hohtola A. Effect of latitude on flavonoid biosynthesis in plants. Plant, Cell Environ. 2010;33(8):1239–47.
13. Wang SY, Zheng W. Effect of plant growth temperature on antioxidant capacity in strawberry. J Agric Food Chem. 2001;49(10):4977–82.
14. Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. Oxid Med Cell Longev. 2009;2(5):270–8.
15. Guasch-Ferré M, Merino J, Sun Q, Fitó M, Salas-Salvadó J. Dietary Polyphenols, Mediterranean Diet, Prediabetes, and Type 2 Diabetes: A Narrative Review of the Evidence. Oxid Med Cell Longev. 2017;2017.

16. Spencer JPE. Proceedings of the Third International Scientific Symposium on Tea and Human Health : Role of Flavonoids in the Diet Metabolism of Tea Flavonoids in the Gastrointestinal Tract 1 , 2. J Nutr [Internet]. 2003;133(27):3255–61. Available from: http://www.ncbi.nlm.nih.gov/pubmed/17073577

17. Kris-Etherton PM, Keen CL. Evidence that the antioxidant flavonoids in tea and cocoa are beneficial for cardiovascular health. Curr Opin Lipidol. 2002;13(1):41–9.

18. Williams CA, Grayer RJ. Anthocyanins and other flavonoids. Nat Prod Rep. 2004;21(4):539–73.

19. Ahn-Jarvis JH, Parihar A, Doseff AI. Dietary flavonoids for immunoregulation and cancer: Food design for targeting disease. Antioxidants. 2019;8(7):1–30.

20. El Haouari M, A. Rosado J. Modulation of Platelet Function and Signaling by Flavonoids. Mini-Reviews Med Chem. 2011;11(2):131–42.

21. Devine CM, Connors M, Bisogni CA, Sobal J. Life-course influences on fruit and vegetable trajectories: Qualitative analysis of food choices. J Nutr Educ Behav. 1998;30(6):361–70.

22. Booth SL, Sallis JF, Ritenbaugh C, Hill JO, Birch LL, Frank LD, et al. Environmental and societal factors affect food choice and physical activity: Rationale, influences, and leverage points. Nutr Rev. 2001;59(3 II):S21–36.

23. Drewnowski A, Darmon N. Symposium : Modifying the Food Environment : Energy Density , Food Costs , and Portion Size Food Choices and Diet Costs : an Economic Analysis 1 , 2. J Nutr. 2005;(February):900–4.

24. Ock KC, Sang JC, Song WO. Estimated dietary flavonoid intake and major food sources of U.S. adults. J Nutr. 2007;137(5):1244–52.

25. Zamora-Ros R, Knaze V, Luján-Barroso L, Slimani N, Romieu I, Fedirko V, et al. Estimated dietary intakes of flavonols, flavanones and flavones in the European Prospective Investigation into Cancer and Nutrition (EPIC) 24 hour dietary recall cohort. Br J Nutr. 2011;106(12):1915–25.

26. Jun S, Shin S, Joung H. Estimation of dietary flavonoid intake and major food sources of Korean adults. Br J Nutr. 2016;115(3):480–9.

27. Zhang Y, Li Y, Cao C, Cao J, Chen W, Zhang Y, et al. Dietary flavonol and flavone intakes and their major food sources in Chinese adults. Nutr Cancer. 2010;62(8):1120–7.

28. Somerset SM, Johannot L. Dietary flavonoid sources in Australian adults. Nutr Cancer. 2008;60(4):442–9.

29. Grosso G, Stepaniak U, Topor-Madry R, Szafraniec K, Pajak A. Estimated dietary intake and major food sources of polyphenols in the Polish arm of the HAPIEE study. Nutrition [Internet]. 2014;30(11–12):1398–403. Available from: http://dx.doi.org/10.1016/j.nut.2014.04.012

30. Otaki N, Kimira M, Katsumata SI, Uehara M, Watanabe S, Suzuki K. Distribution and major sources of flavonoid intakes in the middle-aged Japanese women. J Clin Biochem Nutr. 2009;44(3):231–8.

31. van Erp-Baart M-AJ, Brants HAM, Kiely M, Mulligan A, Turrini A, Sermoneta C, et al. Isoflavone intake in four different European countries: the VENUS approach. Br J Nutr. 2003;89(S1):S25–30.

32. Ness AR, Powles JW. Fruit and vegetables, and cardiovascular disease: A review. Int J Epidemiol. 1997;26(1):1–13.

33. De Groot H, Rauen U. Tissue injury by reactive oxygen species and the protective effects of flavonoids. Fundam Clin Pharmacol. 1998;12(3):249–55.

34. Of E, Flavonoids P, Immune ON, Function IC. Effect of Plant Flavonoids on Immune. Adv Exp Med Biol 1998;439175-82. 1998;439:175–82.

35. Vinson JA. Flavonoids in foods as in vitro and in vivo antioxidants. Adv Exp Med Biol. 1998;439:151–64.

36. Liu ZQ, Ma LP, Zhou B, Yang L, Liu ZL. Antioxidative effects of green tea polyphenols on free radical initiated and photosensitized peroxidation of human low density lipoprotein. Chem Phys Lipids. 2000;106(1):53–63.

37. Hertog MGL, Feskens EJM, Kromhout D, Hertog MGL, Hollman PCH, Hertog MGL, et al. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet. 1993;342(8878):1007–11.

38. Huxley RR, Neil HAW. The relation between dietary flavonol intake and coronary heart disease mortality: A meta-analysis of prospective cohort studies. Eur J Clin Nutr. 2003;57(8):904–8.

39. Knekt P, Jarvinen R, Reunanen A, Maatela J. Article 8 Ref 21. 1996;312(February):1994–7.

40. Nardini M, Natella F, Scaccini C. Role of dietary polyphenols in platelet aggregation. A review of the supplementation studies. Platelets. 2007;18(3):224–43.

41. Freedman JE, Parker C, Li L, Perlman JA, Frei B, Ivanov V, et al. Select flavonoids and whole juice from purple grapes inhibit platelet function and enhance nitric oxide release. Circulation. 2001;103(23):2792–8.

42. Landolfi R, Mower RL, Steiner M. Modification of platelet function and arachidonic acid metabolism by bioflavonoids. Structure-activity relations. Biochem Pharmacol. 1984;33(9):1525–30.

43. Bucki R, Pastore TJJ, Giraud F, Sulpicejand JC, Janmey PA. Flavonoid inhibition of platelet procoagulant activity and phosphoinositide synthesis. J Thromb Haemost. 2003;1(8):1820–8.

44. Bhat EA, khan J, Sajjad N, Ali A, Aldakeel FM, Mateen A, et al. SARS-CoV-2: Insight in genome structure, pathogenesis and viral receptor binding analysis – An updated review. Int Immunopharmacol [Internet]. 2021;95(November 2020):107493. Available from: https://doi.org/10.1016/j.intimp.2021.107493

45. Gale AJ. Continuing Education Course #2: Current Understanding of Hemostasis. Toxicol Pathol. 2011;39(1):273–80.

46. Widmaier, Eric P.; Raff, Hershel; Strang KT. Vander's Human Physiology: The Mechanisms of Body Function. Fourteenth. New York, NY: McGraw-Hill Education; 2016.

47. Dahlbäck B. Blood coagulation. Lancet. 2000;355(9215):1627–32.

48. Lane DA, Philippou H, Huntington JA. Directing thrombin. Blood. 2005;106(8):2605–12.

49. Toschi V, Lettino M. Inhibitors of propagation of coagulation: Factors V and X. Br J

Clin Pharmacol. 2011;72(4):563–80.

50. Nam GS, Lee KS, Nam KS. Morin hydrate inhibits platelet activation and clot retraction by regulating integrin αIIbβ3, TXA2, and cAMP levels. Eur J Pharmacol [Internet]. 2019;865(October):172734. Available from: https://doi.org/10.1016/j.ejphar.2019.172734

51. Hostetler GL, Ralston RA, Schwartz SJ. Flavones : Food Sources , Bioavailability ,. 2017;423–35.

52. Choi JH, Kim YS, Shin CH, Lee HJ, Kim S. Antithrombotic Activities of Luteolin In Vitro and In Vivo. J Biochem Mol Toxicol. 2015;29(12):552–8.

53. Om H, El-Naggar ME, El-Banna M, Fouda MMG, Othman SI, Allam AA, et al. Combating atherosclerosis with targeted Diosmin nanoparticles-treated experimental diabetes. Invest New Drugs. 2020;38(5):1303–15.

54. Paredes MD, Romecín P, Atucha NM, O'Valle F, Castillo J, Ortiz MC, et al. Beneficial effects of different flavonoids on vascular and renal function in L-NAME hypertensive rats. Nutrients. 2018;10(4):1–15.

55. Cheng C, Zhang H, Li Y, Zhou Y, Lu W, Yao L. The effect of Diosmin on the blood proteome in a rat model of venous thrombosis. Int J Biol Macromol [Internet]. 2017;104:778–87. Available from: http://dx.doi.org/10.1016/j.ijbiomac.2017.06.045

56. Zheng Y, Zhang R, Shi W, Li L, Liu H, Chen Z, et al. Metabolism and pharmacological activities of the natural health-benefiting compound diosmin. Food Funct. 2020;11(10):8472–92.

57. Singh B, Sidiq T, Joshi P, Jain SK, Lawaniya Y, Kichlu S, et al. Anti-inflammatory and immunomodulatory flavones from Actinocarya tibetica Benth. Nat Prod Res. 2013;27(23):2227–30.

58. Qiu Y, Cong N, Liang M, Wang Y, Wang J. Systems Pharmacology Dissection of the Protective Effect of Myricetin Against Acute Ischemia/Reperfusion-Induced Myocardial Injury in Isolated Rat Heart. Cardiovasc Toxicol. 2017;17(3):277–86.

59. Meng Z, Wang M, Xing J, Liu Y, Li H. Myricetin ameliorates atherosclerosis in the low-density-lipoprotein receptor knockout mice by suppression of cholesterol accumulation in macrophage foam cells. Nutr Metab. 2019;16(1):1–9.

60. Chen S-Y, Chu C-C, Jiang C-L, Duh P-D. The Vasodilating Effect and Angiotensin Converting Enzyme Inhibition Activity of Three Dietary Flavonols: Comparsion between Myricetin, Quercetin and Morin, *in vitro*. J Food Nutr Res. 2019;7(5):347–54.

61. Gaspar RS, Da Silva SA, Stapleton J, De Lima Fontelles JL, Sousa HR, Chagas VT, et al. Myricetin, the main flavonoid in syzygium cumini leaf, is a novel inhibitor of platelet thiol isomerases PDI and ERp5. Front Pharmacol. 2020;10(January):1–15.

62. Lescano CH, de Lima FF, Mendes-Silvério CB, Justo AFO, Baldivia D da S, Vieira CP, et al. Effect of polyphenols from Campomanesia adamantium on platelet aggregation and inhibition of cyclooxygenases: Molecular docking and in vitro analysis. Front Pharmacol. 2018;9(JUN):1–13.

63. Bijak M, Ponczek MB, Nowak P. Polyphenol compounds belonging to flavonoids inhibit activity of coagulation factor X. Int J Biol Macromol [Internet]. 2014;65:129–35. Available from: http://dx.doi.org/10.1016/j.ijbiomac.2014.01.023

64. Choi JH, Park SE, Kim SJ, Kim S. Kaempferol inhibits thrombosis and platelet

activation. Biochimie [Internet]. 2015;115:177–86. Available from: http://dx.doi.org/10.1016/j.biochi.2015.06.001

65. Guerrero L, Castillo J, Quiñones M, Garcia-Vallvé S, Arola L, Pujadas G, et al. Inhibition of Angiotensin-Converting Enzyme Activity by Flavonoids: Structure-Activity Relationship Studies. PLoS One. 2012;7(11):1–11.

66. Nijveldt RJ, Nood E van, Hoorn DE van, Boelens PG, Norren K van, Leeuwen PA van. Flavonoids a review of probable mechanisms of action. Am J Clin Nutr. 2012;74(4):418–25.

67. Jin YR, Han XH, Zhang YH, Lee JJ, Lim Y, Chung JH, et al. Antiplatelet activity of hesperetin, a bioflavonoid, is mainly mediated by inhibition of PLC-γ2 phosphorylation and cyclooxygenase-1 activity. Atherosclerosis. 2007;194(1):144–52.

68. Yu HY, Park SW, Chung IM, Jung YS. Anti-platelet effects of yuzu extract and its component. Food Chem Toxicol [Internet]. 2011;49(12):3018–24. Available from: http://dx.doi.org/10.1016/j.fct.2011.09.038

69. Yamamoto M, Jokura H, Suzuki A, Hase T, Shimotoyodome A. Effects of continuous ingestion of hesperidin and glucosyl hesperidin on vascular gene expression in spontaneously hypertensive rats. J Nutr Sci Vitaminol (Tokyo). 2013;59(5):470–3.

70. McGregor L, Bellangeon M, Chignier E, Lerond L, Rousselle C, McGregor JL. Effect of a micronized purified flavonoid fraction on in vivo platelet functions in the rat. Thromb Res. 1999;94(4):235–40.

71. Plotnikov MB, Aliev OI, Sidekhmenova A V., Shamanaev AY, Anishchenko AM, Fomina TI, et al. Dihydroquercetin Improves Microvascularization and Microcirculation in the Brain Cortex of SHR Rats during the Development of Arterial Hypertension. Bull Exp Biol Med. 2017;163(1):57–60.

72. Stenger Moura FC, Perioli L, Pagano C, Vivani R, Ambrogi V, Bresolin TM, et al. Chitosan composite microparticles: A promising gastroadhesive system for taxifolin. Carbohydr Polym [Internet]. 2019;218(April):343–54. Available from: https://doi.org/10.1016/j.carbpol.2019.04.075

73. Vitolins MZ, Anthony M, Burke GL. Soy protein isoflavones, lipids and arterial disease. Curr Opin Lipidol. 2001;12(4):433–7.

74. Ganai AA, Farooqi H. Bioactivity of genistein: A review of in vitro and in vivo studies. Biomed Pharmacother [Internet]. 2015;76:30–8. Available from: http://dx.doi.org/10.1016/j.biopha.2015.10.026

75. Gottstein N, Ewins BA, Eccleston C, Hubbard GP, Kavanagh IC, Minihane A-M, et al. Effect of genistein and daidzein on platelet aggregation and monocyte and endothelial function. Br J Nutr. 2003;89(5):607–15.

76. Cesar PHS, Trento MVC, Konig IFM, Marcussi S. Catechin and epicatechin as an adjuvant in the therapy of hemostasis disorders induced by snake venoms. J Biochem Mol Toxicol. 2020;34(12):1–9.

77. Sajevic T, Leonardi A, Križaj I. Haemostatically active proteins in snake venoms. Toxicon. 2011;57(5):627–45.

78. Cheng IF, Breen K. On the ability of four flavonoids, baicilein, luteolin, naringenin, and quercetin, to suppress the fenton reaction of the iron-ATP complex. BioMetals. 2000;13(1):77–83.

79. Bijak M. Silybin, a Major Bioactive Component of Milk Thistle (Silybum marianum L. Gaernt.)—Chemistry, Bioavailability, and Metabolism. Molecules. 2017;22(11):1–11.

80. Federico A, Dallio M, Loguercio C. Silymarin/Silybin and chronic liver disease: A marriage of many years. Molecules. 2017;22(2).

81. De Caterina R, Husted S, Wallentin L, Agnelli G, Bachmann F, Baigent C, et al. Anticoagulants in heart disease: Current status and perspectives. Eur Heart J. 2007;28(7):880–913.